STUDIES ON THE FERMENTATIVE PRODUCTION OF PYRIMIDINE NUCLEOSIDE DIPHOSPHATE COENZYMES

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ABBREVIATIONS

UDPG	Uridine diphosphate glucose
UDPGal	Uridine diphosphate galactose
UDPAG	Uridine diphosphate N-acetylglucosamine
UDPGA	Uridine diphosphate glucuronic acid
GDPM	Guanosine diphosphate mannose
CDP-choline	Cytidine diphosphate choline
CDP-ethanolamine	Cytidine diphosphate ethanolamine
5'-UMP	Uridine 5'-monophosphate
5'-CMP	Cytidine 5'-monophosphate
5'-GMP	Guanosine 5'-monophosphate
5'-AMP	Adenosine 5'-monophosphate
UDP	Uridine 5'-diphosphate
UTP	Uridine 5'-triphosphate
CDP	Cytidine 5'-diphosphate
CTP	Cytidine 5'-triphosphate
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'-triphosphate
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADH	Reduced nicotinamide adenine dinucleotide
G-1-P	D-Glucose-1-phosphate
Gal-1-P	D-Galactose-1-phosphate
G-6-P	D-Glucose-6-phosphate
Pi	Inorganic phosphate
PPi	Inorganic pyrophosphate

INTRODUCTION

In 1949, Leloir and his collaborators[1-3] first discovered UDPG as a coenzyme of galactose phosphate-glucose phosphate transformation during studies on the galactose metabolism in *Saccharomyces fragilis*. Leloir[4] also demonstrated an enzyme in the yeast catalyzing the interconversion of UDPG to UDPGal. At about the same time as Leloir's group isolated UDPG from yeast, Park and Johnson[5-7] obtained the evidence for the accumulation of UDP-N-acetylhexosamine derivatives in penicillin-treated *Staphylococcus aureus*.

The discovery of these uridine diphosphate compounds by the great pioneering works opened up a new chapter in the metabolism of carbohydrates and biosynthesis of polysaccharides. Over sixty of the similar class of compounds containing different bases and sugars or sugar derivatives, which are commonly defined as sugar nucleotides, have been isolated from natural sources as well as enzymatically synthesized.[8] It has been clearly established that the sugar nucleotides play an important role in transformation of monosaccharides, transglycosylation to give more complex saccharides, and biosynthesis of bacterial cell walls.[8,9]

On the other hand, Kennedy and Weiss[78] first discovered CDP-choline and CDP-ethanolamine as naturally occurring coenzyme forms of phosphorylcholine and phosphorylethanolamine. The biological roles of these cytidine coenzymes in the biosynthesis of phospho-

lipids have been elucidated.[79]

Recent extensive studies of metabolic pathways and biosynthetic mechanisms have come to stress the significance of purine and pyrimidine nucleoside diphosphate coenzymes involved in many biological reactions. The numbers of this class of compounds as well as knowledge concerning their functions and chemical properties have been growing rapidly at the present time. However, in spite of the wide distribution and biological importance of these coenzymes, they have been exclusively obtained, in a limited extent, by chemical and enzymic synthesis or by extraction from microorganisms which contain a small and variable amount of the coenzymes.

Roseman *et al.*[10] synthesized a variety of nucleoside diphosphate coenzymes, *e.g.* UDPG, UDPGal, UDPAG, UDPGA, GDPM, *etc.*, by condensation of respective nucleoside 5'-phosphomorpholidates with phosphomonoester components. Michelson[11] also reported a chemical method for their synthesis using nucleoside 5'-diphenylpyrophosphate.

Leloir and his coworkers, on the other hand, prepared UDPG,[3] UDPAG,[12,13] and GDPM[14] from toluene-autolyzed baker's yeast as starting material by extraction with ethanol. About 20-100 mg of these coenzymes were isolated from 10 kg of baker's yeast by their improved methods.[15] It is reported that a small amount of UDPGal is accumulated in the cells of UDPGal 4-epimeraseless mutants of *Salmonella* and *Escherichia coli* [16,17] grown on galactose. The

method of isolation of UDPGal from these bacterial cells is reported.[18,19] Smith and Mills[20] isolated ^{14}C-UDPG and ^{14}C-UDPAG from acetone powder of *Neurospora crassa* grown on a ^{14}C-acetate medium, and Anderson *et al.*[21] synthesized enzymatically a small amount of ^{14}C-UDPG and ^{14}C-UDPGal from labeled glucose and galactose. CDP-Choline and CDP-ethanolamine were isolated from rat liver and yeast,[78] and were also chemically synthesized by Kennedy.[77]

All these investigations, however, have dealt with the isolation and preparation of a small amount of nucleoside diphosphate coenzymes. Current progresses in the field of flavor-enhancing 5'-nucleotide fermentation in this country have led to the extensive studies on the production and utilization of various nucleotides, nucleosides and their related compounds. Tochikura *et al.*[22] have recently reported that 5'-UMP was converted to UDPG in high yields by ground-cells of baker's yeast and acetone powder of brewer's yeast in the presence of glucose and high concentration of inorganic phosphate. They also observed that large amounts of GDPM were formed from 5'-GMP under the same fermentative conditions with air-dried cells of baker's yeast.[23,24]

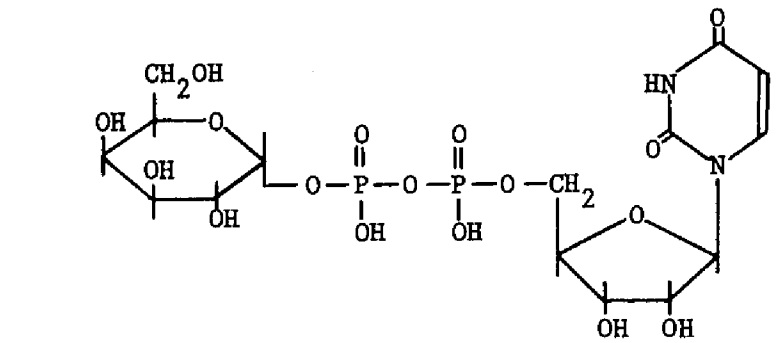
The present study is concerned with the fermentative production and metabolism of biologically important pyrimidine nucleoside diphosphate coenzymes by yeasts. The first chapter deals with a new preparative method of one of the representative uridine coenzymes,

UDPGal, with air-dried cells of *Torulopsis candida* IFO 0768, and its mechanism of UDPGal fermentation. It was described that remarkable amounts of UDPGal were formed from 5'-UMP when galactose was incubated, in the presence of inorganic phosphate, with air-dried cells of the yeast which was grown on a galactose or lactose medium. The various factors affecting UDPGal fermentation by the yeast were also investigated. It was found that the yield of UDPGal was remarkably influenced by the water content of the air-dried cells used as enzyme source, the relationship between the water content and enzyme activities responsible to UDPGal synthesis being studied. The author also elucidated the mechanism of UDPGal fermentation by the yeast with air-dried cells and cell-free system. It was suggested that the efficient accumulation of UDPGal might be brought about by a concerted inhibition of UDPGal 4-epimerase activity by 5'-UMP and galactose.

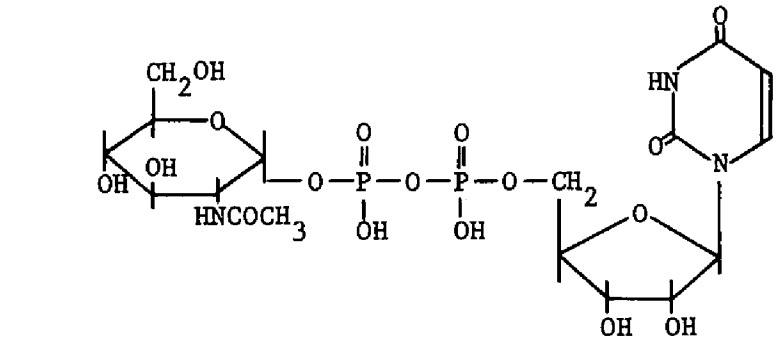
In the second chapter, the author has investigated the fermentative production of UDPAG with air-dried cells of baker's yeast. It was clarified that UDPAG was accumulated in large amounts from 5'-UMP and glucosamine under the fermentative conditions of hexose by the yeast. The fundamental reaction conditions by the yeast as well as the distribution of UDPAG forming activity among other strains of yeast were investigated.

By the application of the fermentative methods of UDPGal and

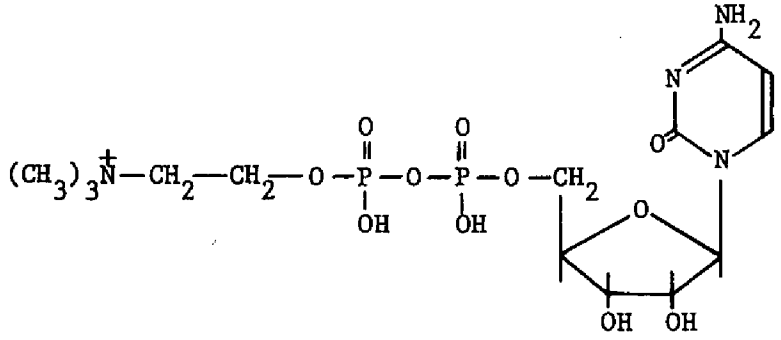
UDPAG studied in the preceding chapters, the author described in the third chapter the preparative methods for CDP-choline and CDP-ethanolamine from 5'-CMP with air-dried cells of brewer's yeast as enzyme source. It was observed that large amounts of CDP-choline were accumulated when the air-dried cells of brewer's yeast were incubated with 5'-CMP and phosphorylcholine in the presence of glucose and inorganic phosphate. The effects of several factors affecting CDP-choline fermentation and the distribution of its forming activity among various yeasts were investigated. It was also found that CDP-ethanolamine was formed when phosphorylcholine was replaced by phosphorylethanolamine in the reaction system for CDP-choline fermentation.



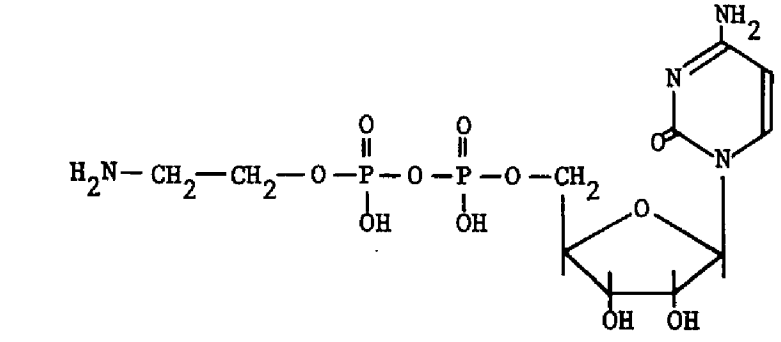
UDP-Galactose



UDP-N-Acetyl-glucosamine



CDP-Choline



CDP-Ethanolamine

Chemical Structure of Uridine and Cytidine Diphosphate Coenzymes

Chapter I. Fermentative Production of UDP-Galactose by *Torulopsis candida*

Section 1. UDP-Galactose Formation from 5'-UMP

INTRODUCTION

Since UDPGal was isolated from *Saccharomyces fragilis*,[4] this uridine coenzyme has been shown to be an important intermediate on the Leloir pathway of galactose metabolism.[25]

The preparation of UDPGal from Gal-1-P with calf liver Gal-1-P uridylyltransferase has been reported.[21,26] When UDPGal 4-epimerase-less mutants of *Salmonella* and *Escherichia coli* were grown on galactose, a small amount of UDPGal was accumulated in the bacterial cells,[16,17] and methods of isolation of UDPGal from such cells were reported.[18,19] These investigations, however, have dealt exclusively with the isolation of only small amount of UDPGal.

Recently, Tochikura *et al.*[27] have reported that several nucleoside 5'-monophosphates were phosphorylated to nucleoside 5'-diphosphates and nucleoside 5'-triphosphates by yeast enzyme preparations under the fermentative conditions of glucose in the presence of high concentration of inorganic phosphate. They also observed that when 5'-UMP or 5'-GMP was incubated under the same reaction conditions, UDPG[22] or GDPM,[23,24] a corresponding nucleotide sugar, was accumulated in the reaction solution in good yields.

This work[28] was undertaken to produce UDPGal, an analogous uridine coenzyme to UDPG, from 5'-UMP with air-dried cells of *Torulopsis candida* as enzyme source.

MATERIALS AND METHODS

Materials. 5'-UMP sodium salt was kindly supplied by Takeda Chemical Industries, Ltd., and Tanabe Seiyaku Co., Ltd. UDPG, UDPGal, UTP and UDP were purchased from Sigma Chemical Co. NAD was obtained from Boehringer and Soehne GmbH, Mannheim. All other chemicals were commercial products.

Microorganisms and cultivation. Torulopsis candida IFO 0768 was mainly used as enzyme source for the production of UDPGal. The screening test for UDPGal accumulating activity was performed with other strains of yeast which were capable of utilizing lactose for their growth. All the yeasts were grown on a medium containing 5% galactose or lactose, 0.5% peptone, 0.2% yeast extract, 0.2% KH_2PO_4, 0.2% $(NH_4)_2HPO_4$ and 0.1% $MgSO_4 \cdot 7H_2O$. The cultivation was carried out at 28°C for 24 to 48 hr on a reciprocal shaker with 2 liter-shaking flask containing 500 ml of the above medium. The cells harvested by centrifugation were washed three times with tap water and air-dried at room temperature for 24 to 48 hr with an electric fan, followed by desiccation overnight under reduced pressure over NaOH or P_2O_5. The air-dried cells were kept at -20°C until used.

Enzyme preparations. Acetone-dried cells and ground cells of *T. candida* were prepared as follows. About 10 g of wet cells were suspended in small volume of water and the suspension was put into 200 ml of -20°C acetone and stirred for 20 min. The cells were collected by centrifugation, washed with ether and dried in a desiccator under reduced pressure. The acetone-dried cells were stored at -20°C until used. Ten grams of dried cells were ground in a cooling mortar for 20 min with 20 g of sea sand and appropriate amount of 0.05 M phosphate buffer (pH 7.0). It was then suspended into 30 ml of the same buffer, followed by decantation to remove sea sand. The supernatant cell suspension was used as ground cells.

UDPG dehydrogenase was purified from acetone powder of bovine liver according to the method of Wilson.[29] The acetone saturated fraction (fraction IV) was used for the enzymic assay of UDPG throughout this work. UDPGal 4-epimerase was partially purified from *Saccharomyces fragilis* by the method of Maxwell and Robichon-Szulmajster.[30]

Reaction method. The composition of standard reaction mixture for UDPGal formation is shown in Table I. Unless otherwise stated, the dried cells of *T. candida* grown on the lactose medium were used as enzyme source. The reaction was carried out at 28°C by continuous shaking in a test tube. The reaction was terminated by immersing the tube in boiling water for 3 min, and cooled. The cell debris was

TABLE I. STANDARD REACTION SYSTEM

	per ml
5'-UMP sodium salt	20 μmoles
D-Galactose	200 μmoles
Potassium phosphate buffer (pH 7.0)	200 μmoles
$MgSO_4 \cdot 7H_2O$	12 μmoles
Air-dried cells of *Torulopsis candida*	100 mg

Total volume 2.5 ml

removed by centrifugation and the supernatant solution was submitted to analysis.

Analyses. Uridine nucleotides and nucleoside were determined by paper chromatography. Aliquot of the reaction mixture was spotted on Toyo Filter Paper No. 53 and developed with a solvent system consisted of 95% ethanol-M ammonium acetate (7.5:3, pH 7.5).[3] Ultraviolet absorbing spots were cut off and uridine compounds were eluted with 5 ml of 0.1 N HCl at 30°C for 15 hr. The absorbancy at 260 mμ was measured by Hitachi-Perkin-Elmer spectrophotometer Model 139, and the amounts of each compound were calculated based on a molar absorbancy index for uridine of 9.9×10^3.

UDPG was determined enzymatically with UDPG dehydrogenase by the method of Strominger *et al.*[31] with a slight modification. The assay mixture contained 1.5 μmoles of NAD, 250 μmoles of glycine buffer (pH 8.6), excessive amounts of UDPG dehydrogenase (0.2-0.3 ml of fraction IV), and a sample to be assayed in a total volume of

3 ml. The reaction was initiated in a quarz cell by the addition of the sample containing 0.05-0.1 μmole of UDPG. The optical density at 340 mμ was followed until no more increase in the absorbancy was observed. The amounts of UDPG were calculated from E_{340} equivalent to that of reduced pyridine nucleotide formed.

UDPGal was determined as the difference between total UDP-sugar (UDPG + UDPGal) determined by paper chromatography and UDPG assayed enzymatically, since these two uridine coenzymes gave the same *Rf* value on a paper chromatogram with the above solvent.

The reaction products were separated by column chromatography with Dowex 1 x 2 (Cl^- form) by the method of Cohn and Carter.[32] Paper chromatography of hexoses was carried out with a solvent system of ethyl acetate-water-pyridine (4:4:2, v/v),[33] and aniline hydrogen phthalate reagent[34] was used for their detection. Hexoses were also identified by colorimetric method with carbazole-sulfuric acid reagent.[35] Reducing sugar was determined by the method of Somogyi.[36]

RESULTS

UDPGal formation from 5'-UMP and D-galactose

After 5'-UMP and galactose were aerobically incubated, as shown in Table I, with air-dried cells of *T. candida* grown on the galactose medium, the reaction products were analyzed by paper chromatography and enzymic assay. As is shown in Fig. 1, a rapid consumption of

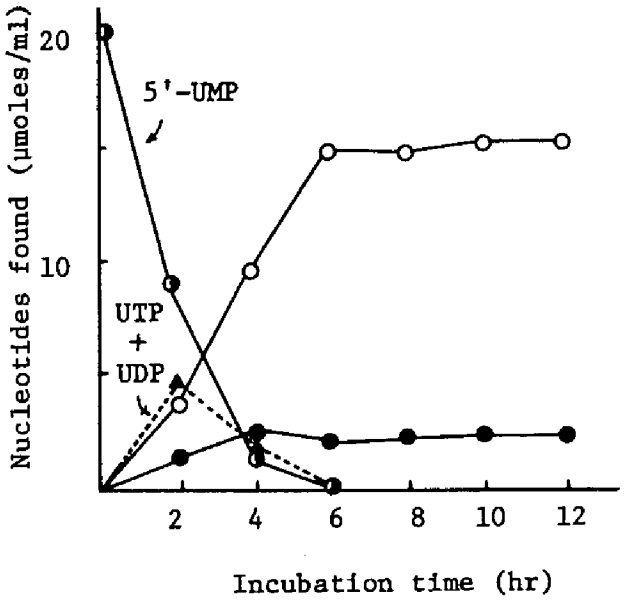


Fig. 1. Formation of Sugar Nucleotides by Galactose-Grown *T. candida*.

The reaction system was described in Table I. Galactose-grown cells were used as enzyme source.

—○— Total UDP-hexose determined by paper chromatography

—●— UDPG determined enzymatically by UDPG dehydrogenase

5'-UMP occurred in the earlier period of the reaction with formation of a small amount of UDP and UTP. Subsequently, UDP and UTP were decreased, followed by the accumulation of a compound whose *Rf* value was identical with that of authentic UDPG in 95% ethanol-M ammonium acetate (7.5:3, pH 7.5) system. But the amount of UDPG assayed with UDPG dehydrogenase was found to be much smaller than

that determined by paper chromatography. The result will suggest that another compound than UDPG having the same *Rf* value in the above solvent may be accumulated in the reaction mixture.

Then an aliquot of the reaction mixture was incubated with excessive UDPG dehydrogenase and NAD to oxidize completely a small amount of UDPG present (Fig. 2). When UDPGal 4-epimerase that catalyzes the conversion of UDPGal to UDPG was added to the incubation mixture at the time indicated by the arrow, a further increase in the optical density at 340 mμ occurred.

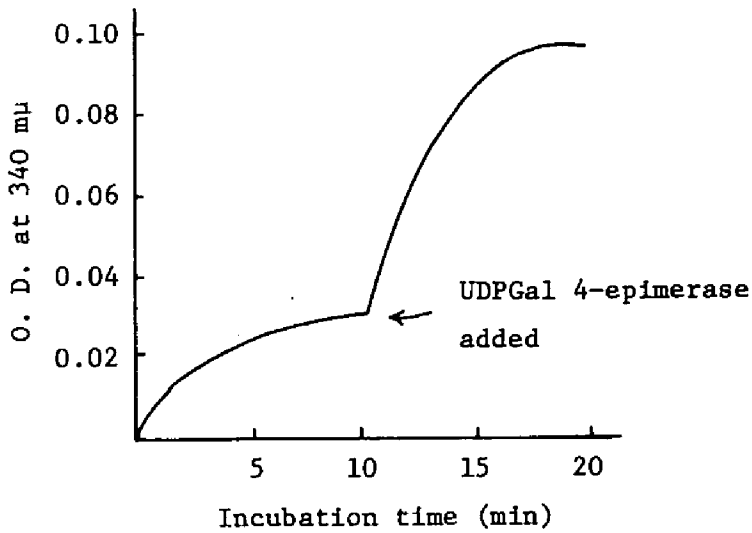


Fig. 2. Biological Activity of the Reaction Product by UDPG Dehydrogenase-UDPGal 4-Epimerase System.

The reaction mixture contained 0.5 μmole of NAD, 0.57 mg of UDPG dehydrogenase, 200 μmoles of glycine buffer (pH 8.6) and aliquot of the reaction solution in a total volume of 2.7 ml. The reaction was carried out at room temperature.

From these observations, it was strongly suggested that fairly large amounts of UDPGal were formed under the reaction condition described in Table I.

Isolation and identification of UDPGal

A large scale incubation was carried out in order to isolate and identify UDPGal. The standard reaction system (Table I) was used except that the reaction was carried out in three 2 liter-shaking flasks, each of which contained 500 ml of the reaction mixture. After 20 hr incubation by continuous shaking at 28°C, the reaction was terminated by immersing flasks in boiling water for 15 min and cell debris was centrifuged off. The supernatant solution was adjusted to pH 3.8 with 1 N HCl and the precipitate was discarded by centrifugation. To the clear supernatant solution, was added active carbon to adsorb nucleotides and stirred for hours. Then the active carbon was collected by filtration and the adsorbed nucleotides were eluted with 50% ethanol solution containing 5% NH_4OH, and then applied on a column of Dowex 1 x 2 (Cl^- form).

The elution pattern of the nucleotides is shown in Fig. 3. The main uridine derivative was eluted with 0.01 N HCl and 0.20 M NaCl. To this fraction was added active carbon and the adsorbed nucleotide was eluted with ammoniacal ethanol solution. The eluate was concentrated under reduced pressure at 30°C and lyophilized. Finally about 6.0 g of UDPGal was obtained as diammonium salt from

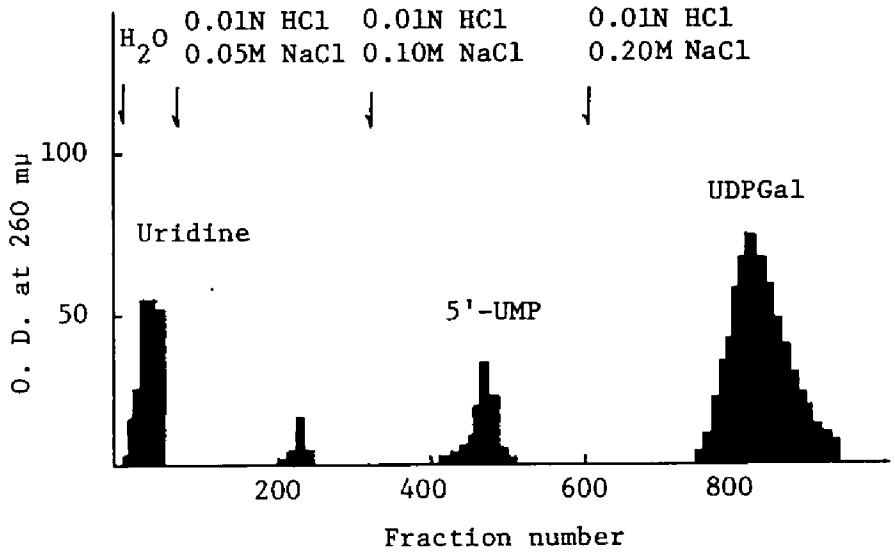


Fig. 3. Separation of UDPGal by Ion Exchange Column Chromatography.

Resin: Dowex 1 x 2 (Cl^- form), 200-400 mesh, 19.6 cm^2 x 52 cm. One fraction: 20 ml. Elution speed: 10 ml/min.

13.2 g of 5'-UMP sodium salt.

The isolated nucleotide was found to have E_{max} at 262 mμ and E_{min} at 230 mμ in 0.01 N HCl, the absorption spectrum being identical with that of uridine. Paper chromatogram of the isolate and its acid-hydrolyzate is shown in Fig. 4. The *Rf* value of the isolate was almost identical with that of authentic UDPGal, and UDP was liberated from the isolate when it was hydrolyzed in 0.01 N HCl at 100°C for 15 min. One mole of UDP was released from one mole of the isolate by the acid-hydrolysis. Furthermore, a hexose was liberated by the hydrolysis, whose *Rf* value was identical with that of authentic

A: Authentic samples

B: Acid-hydrolyzate (0.01N HCl, 100°C, 15 min)

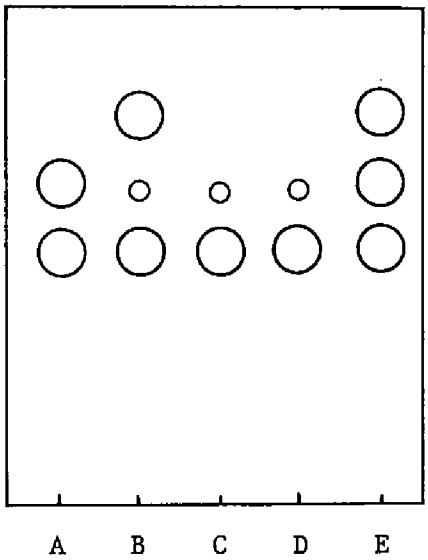
C: Isolated sample

Solvent: 95% ethanol-M-ammonium acetate (7.5:3, pH 7.5)

Fig. 4. Paper Chromatogram of Isolated UDPGal and Its Acid-Hydrolyzate

galactose (Fig. 5). The amount of galactose released was determined by the method of Somogyi.[36] About one mole of galactose was liberated from one mole of the isolate. The absorption spectrum of the colored product of the hexose liberated by the hydrolysis was identical with that of authentic galactose when it was developed by carbazole-H_2SO_4 reaction[35] (Fig. 6).

The NMR spectrum of the isolated nucleotide is shown in Fig. 7.



A: Sample + glucose
B: Sample + mannose
C: Sample + galactose
D: Sample
E: Glucose + mannose + galactose
Solvent: ethyl acetate-H_2O-pyridine (4:4:2, v/v)
Sample: acid-hydrolyzate (0.01N HCl, 100°C, 15 min)

Fig. 5. Paper Chromatogram of Sugar Moiety of Isolated UDPGal.

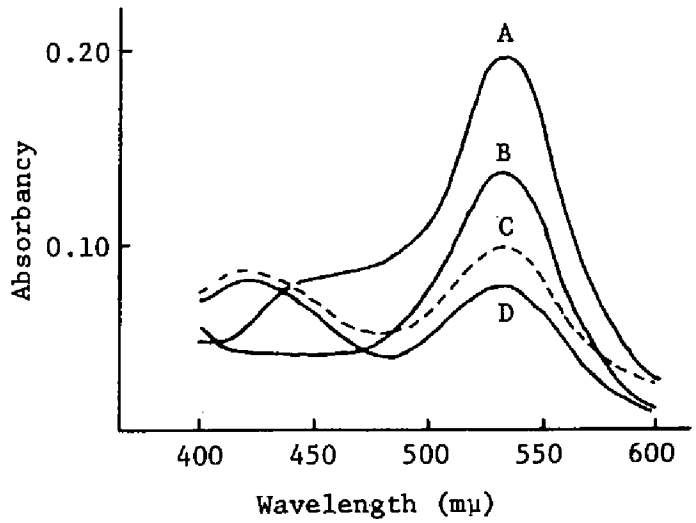


Fig. 6. Absorption Spectra of Colored Products of Sugars by Carbazole-H_2SO_4 Reaction.

A: fructose, B: glucose, C: sugar moiety of isolated UDPGal, D: galactose

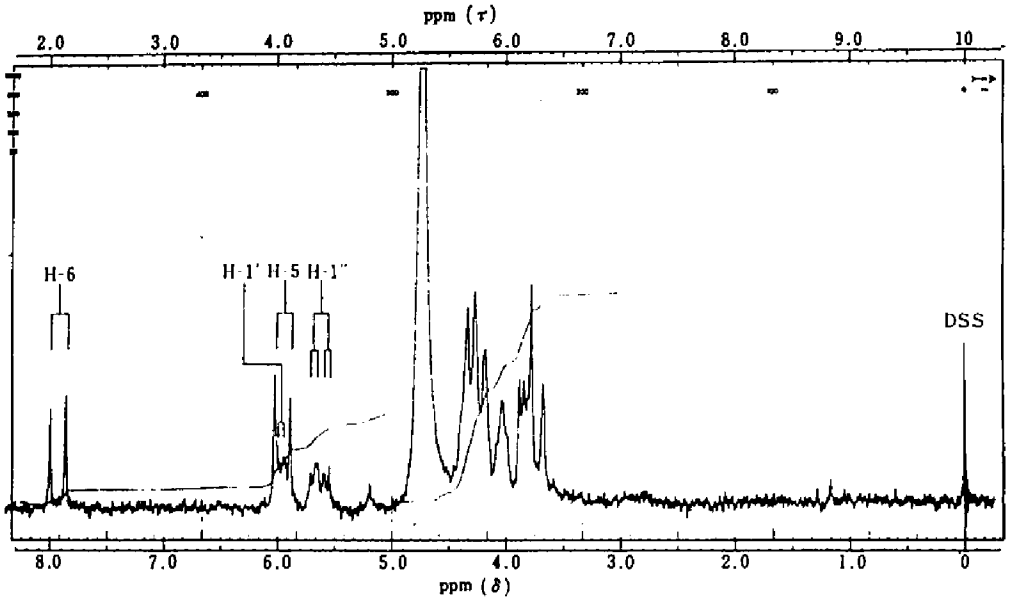


Fig. 7. NMR Spectrum of Isolated UDPGal.

The spectrum was recorded on a Varian A-60 spectrometer at 60 MHz in D_2O with DSS as internal standard.

The signals were assigned as follows: δ 7.92 d ($J_{6,5}$ = 8.0 Hz) (H-6, one proton), δ 5.96 d ($J_{5,6}$ = 8.0 Hz) (H-5, one proton), δ 5.97 d ($J_{1',2'}$ =3.5 Hz) (H-1', one proton), δ 5.63 q ($J_{1'',2''}$ = 3.0 Hz, $J_{1'',p}$ = 7.0 Hz) (H-1", one proton), δ 4.33-3.68 m (methine and methylen's protons of D-ribose and D-galactose, 11 protons). These data were in good agreement with those of UDPGal.

From the results described so far, it was concluded that the isolated nucleotide was UDPGal. It was found by enzymic and chromatographical analyses that the isolated UDPGal was contaminated by small amounts of UDPG (5.9%) and UDP (3.7%).

Effect of various factors on UDPGal fermentation

Phosphate buffer concentration. The optimum concentration of phosphate buffer was found between 0.1 M and 0.4 M, the maximum yield of UDPGal being about 65% based on 5'-UMP. With increasing phosphate concentration, UDP and UTP were increased, while the consumption of 5'-UMP became slower. At lower phosphate concentration (0.1 M and 0.2 M), UDPG which was accumulated in the early period of the reaction was gradually decreased in the later stage of incubation, whereas higher phosphate concentration (0.4 M and 0.8 M) caused a gradual increase of UDPG.

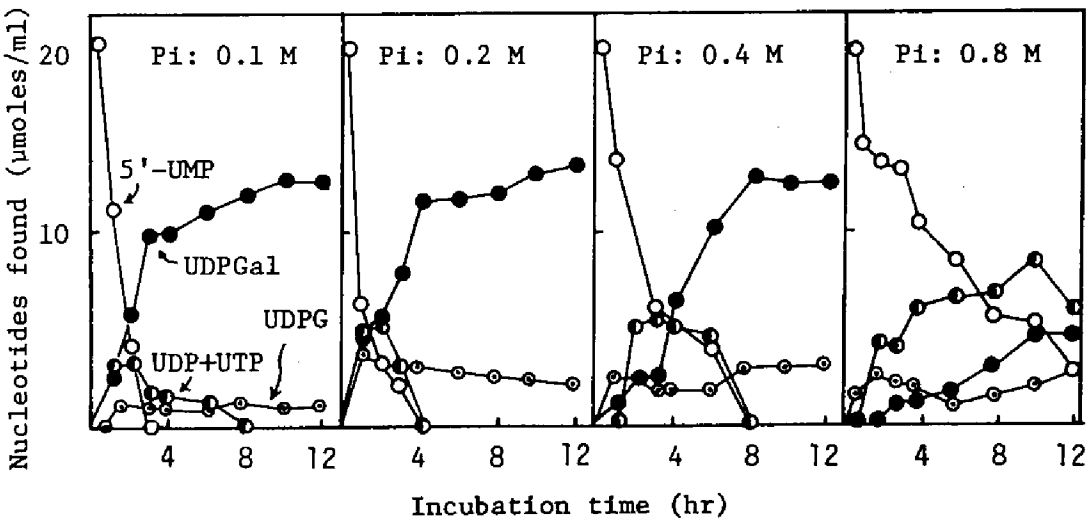


Fig. 8. Effect of Phosphate Concentration on UDPGal Formation by Lactose-Grown *T. candida*.

The reaction system was described in Table I except that phosphate buffer concentration was varied.

5'-UMP concentration. The effect of 5'-UMP concentration is shown in Fig. 9. The maximum yields of UDPGal at the concentrations of 10, 20, 40 and 60 μmoles/ml of 5'-UMP were 88.0%, 80.3%, 79.4% and 69.8%, respectively. Sometimes the yields of UDPGal varied from 60% to 80% in the standard reaction system (5'-UMP 20 μmoles/ml) depending on the preparation of air-dried cells. It can also be seen that UDPGal once accumulated is very stable even after a long incubation. On the other hand, UDPG which was accumulated in the earlier stage of the reaction tended to decrease gradually in the later stage of the reaction.

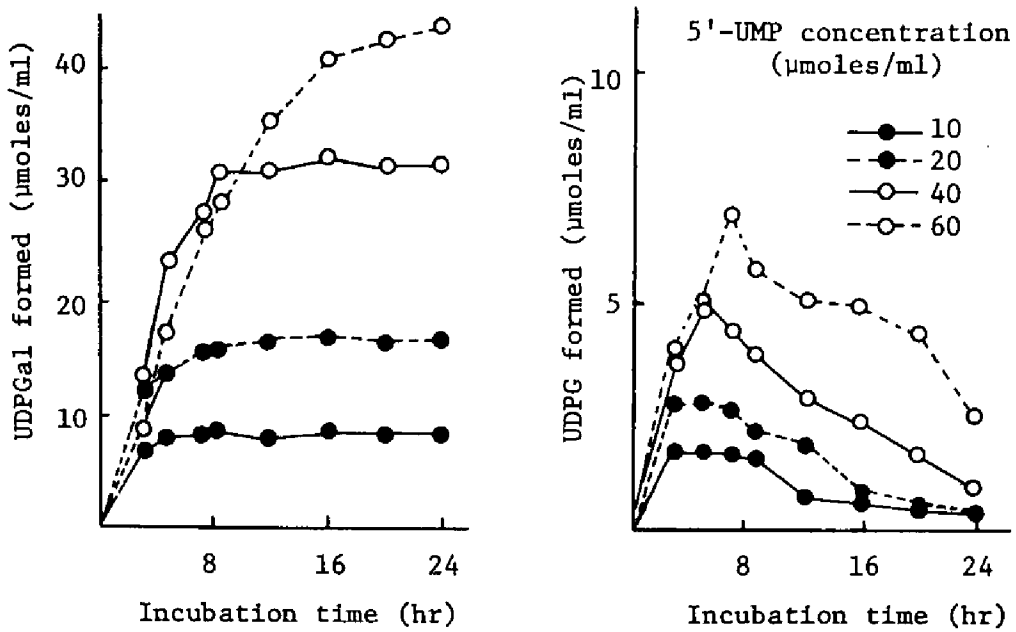


Fig. 9. Effect of 5'-UMP Concentration on Formation of UDPGal and UDPG by Lactose-Grown *T. candida*.

The reaction system was described in Table I except that 5'-UMP concentration was varied.

Galactose concentration. As is shown in Fig. 10, the presence of galactose is essential for the fermentative production of UDPGal. The accumulation of UDPGal was delayed by the addition of higher concentration of galactose in the early period of the reaction. About 200 μmoles/ml of galactose was enough to ensure a maximum yield of UDPGal. It is of great interest that in the absence of galactose large amounts of UDPG were formed instead of UDPGal (Fig. 11). This result would suggest that UDPG may be biosynthesized from G-1-P derived from endogenous carbohydrates such as intracellular glycogen of the dried cells used as enzyme source.

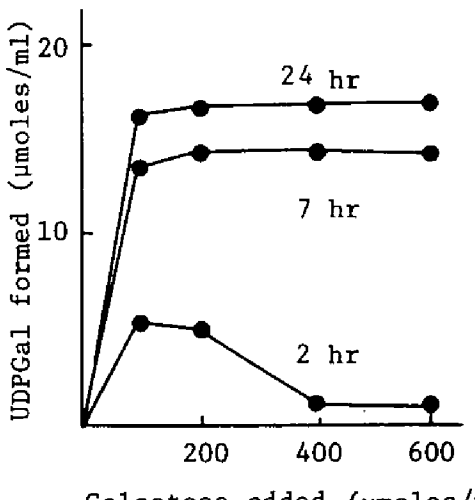


Fig. 10. Effect of Galactose Concentration on UDPGal Formation by Lactose-Grown *T. candida*.

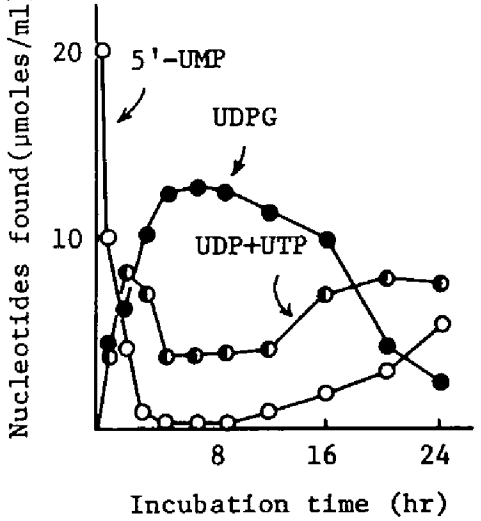


Fig. 11. UDPG Formation in the Absence of Galactose by Lactose-Grown *T. candida*.

The reaction system in Figs. 10 and 11 was the same as that described in Table I except that galactose concentration was varied.

Dried cells concentration. Table II shows that more than 75 mg/ml of the dried cells are required for a maximal formation of UDPGal under the standard reaction condition. UDPGal formation was delayed remarkably at 50 mg/ml of the cells, and no UDP-hexose was formed at 25 mg/ml.

TABLE II. EFFECT OF CELL CONCENTRATION ON UDPGAL FORMATION BY LACTOSE-GROWN *T. candida*

Concentration of dried cells (mg/ml)	Incubation time (hr)	Products and substrate found (μmoles/ml)				
		Uridine	UDPGal	UDPG	5'-UMP	UDP + UTP
100	10	2.0	12.8	2.4	0	0
	15	1.9	12.6	3.5	0	0
75	10	2.0	14.0	2.4	0	0
	15	1.6	13.3	2.9	0	0
50	10	3.3	0	0	11.1	2.1
	15	3.7	9.4	1.0	4.2	2.6
25	10	2.4	0	0	15.1	0
	15	5.0	0	0	13.7	0

The reaction system was described in Table I except that cell concentration was varied.

Effect of sugar substrate. When galactose was replaced by glucose in the standard reaction system, only UDPG was accumulated in good yields (Table III). On the other hand, in the reaction

systems containing galactose, galactose + glucose, and lactose as sugar substrate, UDPGal was mainly accumulated with incubation time against gradual decrease of UDPG.

TABLE III. EFFECT OF SUGAR SUBSTRATE ON UDP-HEXOSE FORMATION BY LACTOSE-GROWN *T. candida*

Sugar substrates added (μmoles/ml)		UDP-hexose formed (μmoles/ml)					
		UDPG			UDPGal		
		4hr	8hr	12 hr	4hr	8hr	12hr
Glucose	400	7.8	10.8	10.0	0	1.1	0.7
Galactose	400	6.1	3.7	1.7	4.2	9.8	12.2
Glucose + Galactose	each 200	5.1	4.7	3.1	5.2	10.5	12.0
Lactose	200	5.4	4.3	2.4	7.3	8.9	9.9

The reaction system was described in Table 1 except that sugar substrates and their concentrations were varied as shown above.

Effect of aeration. An aerobic reaction condition was essential to the fermentative production of UDPGal (Table IV). This observation seems to be consistent with the absence of the fermentative ability of *T. candida.*[37)]

Effect of cell preparations. The effect of various cell preparations on UDPGal formation was investigated (Table V). Not only air-dried cells but also ground cells were found suitable as enzyme sources for the efficient formation of UDPGal. Large amounts of

TABLE IV. EFFECT OF AERATION ON UDPGAL FORMATION BY LACTOSE-GROWN *T. candida*

Diameter of test tube (mm)	Volume of reaction solution (ml)	UDPGal formed (μmoles/ml)	
		Lactose system	Galactose system
16	2.5	0.3*	0.3*
	2.5	12.0	12.6
	5.0	11.5	10.2
	7.5	2.6	3.1
	10.0	0.7	1.4
24	2.5	12.3	11.0
	5.0	13.0	11.2

* Static reaction The reaction system was described in Table I except that lactose was incubated in the lactose system.

TABLE V. EFFECT OF CELL PREPARATIONS ON UDPGAL FORMATION BY LACTOSE-GROWN *T. candida*

Cell preparations	Incubation time (hr)	Products and substrate found (μmoles/ml)			
		Uridine	UDPGal	UDPG	5'-UMP
Air-dried cells	6	1.1	11.6	2.3	0
	8	1.8	12.0	2.2	0
	10	1.4	13.0	2.1	0
Ground cells	6	1.7	11.7	3.2	0
	8	1.4	11.1	3.6	0
	10	1.0	11.8	1.3	0
Acetone-dried cells	6	17.1	0	0	0
	8	16.2	0	0	0
	10	15.8	0	0	0
Intact cells	6	0	0	0	19.6
	8	0	0	0	19.5
	10	0	0	0	19.6

The reaction system was described in Table I except that various cell preparations were used.

uridine were accumulated with acetone-dried cells, whereas 5'-UMP was unchanged with intact cells.

Distribution of UDPGal accumulating activity in yeasts

Nineteen strains of yeasts which are able to assimilate lactose were selected for the screening test. The result is shown in Table VI. It was found that *Saccharomyces lactis*, *Brettanomyces claussenii*, *Torulopsis candida* (three strains), *T. sphaerica*, *Candida intermedia* and *C. pseudotropicalis* (IFO 0882) accumulated both UDPGal and UDPG in various amounts. Three strains of *T. candida* accumulated UDPGal in good yields in the later stage of the reaction. UDPG was formed in small amounts in later stage of the reaction. In other strains, UDPGal was gradually decreased with incubation time accompanied by the increase of UDPG. The other 11 strains could not produce UDPGal under the reaction condition. Most of them converted 5'-UMP to uridine in large amounts. It is of interest to note that *Saccharomyces fragilis*, from which UDPGal was first isolated[4], can not produce the uridine coenzyme under the reaction condition.

TABLE VI. DISTRIBUTION OF UDPGAL ACCUMULATING ACTIVITY IN YEASTS

Strains	Reaction time (hr)	Products found (μmoles/ml)					
		UDPGal		UDPG		Uridine	
		A	B	A	B	A	B
Saccharomyces fragilis IFO 0288	4	0	0	0	0	13.3	11.3
	8	0	0	0	0	17.5	14.5
	12	0	0	0	0	19.8	19.1
Saccharomyces lactis IFO 1090	4	5.3	9.3	1.9	2.1	1.9	trace
	8	1.6	14.8	11.4	3.4	2.0	trace
	12	2.1	3.5	9.2	12.4	2.1	trace
Debaryomyces subglobosus IFO 0794	4	0	0	2.8	1.1	trace	trace
	8	0	0	5.9	2.7	trace	trace
	12	0	0	5.9	3.3	trace	trace
Bullera alba IFO 1192	4	0	0	0	0	14.3	14.0
	8	0	0	0	0	17.6	18.1
	12	0	0	0	0	18.3	18.0
Brettanomyces claussenii IFO 0627	4	2.9	6.3	3.6	9.6	7.9	4.5
	8	2.1	2.8	9.9	12.1	7.1	4.5
	12	1.8	3.6	10.1	11.5	7.1	4.8
Torulopsis candida IFO 0380	4	2.8	1.3	4.8	1.7	trace	trace
	8	8.9	2.9	2.6	2.8	trace	trace
	12	13.1	6.5	2.0	1.9	trace	trace
Torulopsis candida IFO 0768	4	3.3	1.8	5.5	2.1	trace	trace
	8	12.6	5.2	4.5	3.0	trace	trace
	12	12.9	8.6	4.4	3.0	trace	trace
Torulopsis candida IFO 0405	4	1.6	0	1.1	0	0	0
	8	3.0	0	1.5	0.7	1.1	0
	12	13.5	2.8	0.9	1.3	1.3	0
Torulopsis sphaerica IFO 0648	4	10.0	11.5	5.1	4.8	trace	0
	8	4.7	6.9	11.7	9.6	trace	0
	12	4.8	7.9	12.6	9.5	trace	0
Candida humicola IFO 0753	4	0	0	0	0	15.2	11.8
	8	0	0	0	0	18.0	14.8
	12	0	0	0	0	18.2	16.4
Candida humicola IFO 0760	4	0	0	0	0	12.2	9.4
	8	0	0	0	0	15.1	11.9
	12	0	0	0	0	16.1	14.4

TABLE VI. DISTRIBUTION OF UDPGAL ACCUMULATING ACTIVITY IN YEASTS (continued)

Strains	Reaction time (hr)	Products found (μmoles/ml)					
		UDPGal		UDPG		Uridine	
		A	B	A	B	A	B
Candida intermedia IFO 0761	4	6.8	2.8	1.1	9.3	trace	trace
	8	5.8	1.9	1.1	10.5	trace	trace
	12	5.4	1.5	1.6	11.0	trace	trace
Candida macedoniensis IFO 0960	4	0	0	0	0	14.0	9.3
	8	0	0	0	0	15.4	11.0
	12	0	0	0	0	15.8	10.7
Candida pseudotropicalis IFO 0885	4	0	0	0	0	17.1	9.4
	8	0	0	0	0	16.8	10.6
	12	0	0	0	0	18.2	12.1
Candida pseudotropicalis IFO 0617	4	0	0	0	0	11.9	11.3
	8	0	0	0	0	17.5	17.4
	12	0	0	0	0	20.0	19.0
Candida pseudotropicalis IFO 0882	4	10.2	8.5	6.7	4.9	trace	trace
	8	0	6.7	8.5	6.0	trace	trace
	12	0	0	1.9	7.5	trace	trace
Candida pseudotropicalis var. *lactosa* IFO 0008	4	0	0	0	0	18.4	17.6
	8	0	0	0	0	19.4	19.1
	12	0	0	0	0	19.5	19.8
Candida pseudotropicalis var. *lactosa* IFO 0432	4	0	0	0	1.6	17.1	13.2
	8	0	0	0	4.4	17.3	13.8
	12	0	0	0	5.5	17.7	13.7
Candida tenuis IFO 0716	4	0	0	0	0	7.9	6.2
	8	0	0	0	0	12.6	10.4
	12	0	0	0	0	14.8	10.8

A: 5'-UMP + D-galactose system, B: 5'-UMP + lactose system
The reaction system was the same as that described in Table I except that 100 mg/ml of dried cells of various yeasts were incubated as enzyme source. D-Galactose (400 μmoles/ml) and lactose (400 μmoles/ml) were incubated in the systems of (A) and (B), respectively.

DISCUSSION

An investigation of the fermentative production of UDPGal from 5'-UMP with air-dried cells of *Torulopsis candida* was performed in this section. UDPGal was accumulated in the yield of 60-80% based on 5'-UMP added. By a large scale incubation, about 6 g of UDPGal could be prepared from 13.2 g of 5'-UMP sodium salt. Little information has been presented concerning the preparation of UDPGal by such fermentative processes as described in this section.

Tochikura *et al.*[27] recently reported that adenosine and 5'-AMP were phosphorylated to ADP and ATP in high yields by various ground and acetone-dried yeast cells under fermentative conditions of glucose. They also observed that 5'-UMP and 5'-GMP were phosphorylated to form UDP, UTP, UDPG, GDP, GTP and GDPM by baker's and brewer's yeast preparations.[22-24] The phosphorylation of nucleoside and nucleotide by those yeasts had been considered to be carried out by ATP which was generated through glycolysis. In this investigation, however, ATP which is needed for the phosphorylation of 5'-UMP and galactose by *T. candida* might be supplied by oxidative phosphorylation connected with respiration, since an aerobic reaction condition was essential to UDPGal formation.

It is known that UDPGal can be synthesized by actions of enzymes such as UDPGal 4-epimerase (UDPG $\rightleftharpoons$ UDPGal), UDPGal pyrophosphorylase (UTP + Gal-1-P $\rightleftharpoons$ UDPGal + PPi), and Gal-1-P uridylyltransferase

(Gal-1-P + UDPG $\rightleftharpoons$ UDPGal + G-1-P). The presence of these adaptive enzymes in *Saccharomyces fragilis* is reported by several workers,[4,38] and their participation in galactose metabolism in company with galactokinase and UDPG pyrophosphorylase has been clarified.[25] The mechanism of formation and accumulation of UDPGal by *T. candida* observed in this work will be discussed in later section.

SUMMARY

An efficient method for the preparation of UDPGal from 5'-UMP by fermentative process was described. UDPGal was accumulated in good yields (60-80%) when 5'-UMP and galactose were aerobically incubated with air-dried cells of *T. candida* grown on galactose or lactose as enzyme source. UDPGal was isolated from the reaction mixture and identified. The effects of several factors on UDPGal fermentation by the yeast were investigated for establishing optimum reaction conditions. The distribution of UDPGal accumulating activity in yeasts capable of utilizing lactose for growth was investigated, and *T. candida* was found to be the most suitable strain for UDPGal fermentation.

Section 2. Effect of Water Content of Air-Dried Cells on UDP-Hexose Fermentation

INTRODUCTION

In the previous section, the author has reported an efficient method for the fermentative production of UDPGal from 5'-UMP and galactose with air-dried cells of *Torulopsis candida* IFO 0768 as enzyme source in the presence of high concentration of inorganic phosphate. During the course of the study of UDPGal fermentation by the yeast, it was found that the production of UDP-hexoses (UDPGal + UDPG) was remarkably influenced by the water content of air-dried cells of the yeast. It was suggested that the changes of UDP-hexose fermentation which was dependent on the water content of the air-dried cells might be related to their enzyme activities which were responsible to UDP-hexose formation.

This section[39] describes the relation between the water content of air-dried cells of *T. candida* and its activity of UDP-hexose formation. The related enzyme activities of the yeast including UDPG pyrophosphorylase, Gal-1-P uridylyltransferase and galactokinase were examined with the dialyzed cell-free extract which was prepared from air-dried cells containing different amounts of water.

MATERIALS AND METHODS

Materials. UDPG and UTP were prepared by the method of Tochikura *et al.*[22] UDPGal was prepared by the method described in the previous section. ATP was purchased from Kokoku Rayon and Pulp Co., Ltd., and G-1-P was purchased from Boehringer and Soehne GmbH, Mannheim. The other chemicals were commercial products. UDPG dehydrogenase was prepared from the acetone powder of bovine liver according to the method of Wilson.[29]

Cultivation of yeast. Torulopsis candida IFO 0768 was grown on a medium containing 5% lactose or glucose, 0.5% peptone, 0.2% yeast extract, 0.2% KH_2PO_4, 0.2% $(NH_4)_2HPO_4$ and 0.1% $MgSO_4 \cdot 7H_2O$. The pH of the medium was 6.2. The cultivation was carried out at 28°C for 24-48 hr on a reciprocal shaker (120 rpm) with 2 liter-shaking flask containing 500 ml of the media. The yeast was also grown on a large scale in a 20 liter-jar fermentor (Marubishi Laboratory Equipment Co., Ltd.) containing 14 liters of the same media at 30°C for 24 hr. An air flow was set at 20 liters/min and agitator speed was 350 rpm under the internal pressure of 0.6 kg/cm^2.

Preparation of dried cells. The yeast cells were harvested by centrifugation and washed three times with tap water. The cells were then spread over a glass plate and dried at room temperature with an electric fan for 24 to 36 hr (air-dried cells). For the preparation of dried cells of lower water content, the air-dried cells were dried again overnight under reduced pressure over P_2O_5.

Preparation of cell-free extract. Five grams of the air-dried cells were suspended in 30 to 50 ml of 0.1 M potassium phosphate buffer (pH 7.0) in a 300 ml-flask and was shaken at 28°C for 4 hr on a reciprocal shaker. The suspension was then centrifuged at 10,000 rpm for 20 min and the supernatant solution was dialyzed at 4°C overnight against 3 liters of 0.01 M potassium phosphate buffer (pH 7.0).

Reaction method for UDP-hexose formation. The reaction system for UDP-hexose formation by dried cells of *T. candida* is shown in Table I. Unless otherwise stated, galactose was incubated with the lactose-grown cells, and glucose with the glucose-grown cells. UDP-Hexoses thus formed were UDPGal and UDPG, respectively, as mentioned in the previous section.

TABLE I. REACTION SYSTEM FOR UDP-HEXOSE FORMATION

	per ml
5'-UMP sodium salt	20 μmoles
Galactose or glucose	200 μmoles
Potassium phosphate buffer (pH 7.0)	200 μmoles
$MgSO_4 \cdot 7H_2O$	12 μmoles
Dried cells of lactose- or glucose-grown *Torulopsis candida*	100 mg

Total volume 2.5 ml, shaking reaction at 28°C

After shaking reaction at 28°C, the reaction tube was put in

boiling water for 3 min, and cell debris was removed by centrifugation. The supernatant solution was employed for UDP-hexose analysis.

Analytical method. UDPG and UDPGal were determined by the method described in the previous section. Protein was estimated by the method of Lowry *et al.*[40] using egg albumin as standard. The water content of dried cells was determined after drying them at 105°C for 24 hr. The number of dead yeast cells was estimated by staining with methylene blue.

Assay of enzyme activities. UDPG pyrophosphorylase was assayed as follows. The reaction mixture contained 10 μmoles of UTP, 20 μmoles of G-1-P, 10 μmoles of $MgCl_2$, 500 μmoles of glycine buffer (pH 8.6) and the requisite amounts of cell-free extract in a total volume of 3 ml. After incubation at 37°C for 15 min, the reaction was stopped by immersing the tube in boiling water for 1 min and the precipitate was centrifuged off. An aliquot of the supernatant solution was analyzed for UDPG by the enzymic assay.

Gal-1-P uridylyltransferase was assayed in a similar way with the incubation mixture containing 10 μmoles of UDPGal, 40 μmoles of G-1-P, 10 μmoles of $MgCl_2$, 500 μmoles of glycine buffer (pH 8.6) and the requisite amounts of cell-free extract in a total volume of 3 ml. UDPG formed was determined enzymatically as mentioned previously. A unit of UDPG pyrophosphorylase or Gal-1-P uridylyltransferase was defined as the amount of enzyme catalyzing the formation of 1 μmole

of UDPG per hour under the above reaction conditions.

Galactokinase was assayed with the incubation mixture containing 5 μmoles of galactose, 10 μmoles of ATP, 10 μmoles of $MgCl_2$, 250 μmoles of Tris buffer (pH 7.5) and the requisite amounts of cell-free extract in a total volume of 3 ml. After incubation at 37°C for 15 min, 0.5 ml of 5% $ZnSO_4$ and 0.3 N $Ba(OH)_2$ were added and mixed. The reducing sugar in the supernatant solution was determined by the method of Somogyi.[36] A unit of the enzyme was defined as the amount of enzyme catalyzing the consumption of 1 μmole of galactose per hour under the reaction condition.

RESULTS AND DISCUSSION

Preparative conditions of dried cells and their UDP-hexose forming activities

The cells of *T. candida* grown on lactose were immediately air-dried under several conditions and their activities of UDP-hexose formation were investigated. As is shown in Table II, the preparative conditions of dried cells seemed to be very important for efficient formation of UDPGal. It was essential for higher yields of UDPGal to use dried-cell preparations which were desiccated as fast as possible, otherwise uridine formation took place.

Then, the relation between water content of the lactose-grown cells and their activity of UDPGal formation was investigated.

TABLE II. EFFECT OF PREPARATIVE CONDITIONS OF LACTOSE-GROWN DRIED CELLS OF *T. candida* ON UDPGAL FORMATION

Preparative conditions of dried cells	UDPGal found (μmoles/ml)	Uridine found (μmoles/ml)
Dried on a large Petri dish (30 cm diameter) in a thin layer	13.6	3.3
Dried on a small Petri dish (9 cm diameter) in a thin layer	13.2	3.6
Dried on the same dish in about 0.5 cm thick and 4 cm across	11.6	4.1
Dried on the same dish in about 1 cm thick and 4 cm across	8.2	6.0
Dried on the same dish in about 1.5 cm thick and 4 cm across	6.1	7.4
Dried on the same dish filled up with wet cells	0	13.3

The wet cells were dried with an electric fan for about 20 hr at room temperature and then kept in a desiccator overnight under reduced pressure. The reaction system was described in Table I in which galactose was incubated as sugar substrate. The reaction time was 8 hr.

As can be seen in Fig. 1, the activity was remarkably affected by the water content of dried cells. The amounts of UDPGal formed by the lactose-grown cells increased rapidly as their water content decreased below 20%. A maximal yield was attained by dried cells containing 5 to 8% water, while no UDPGal was formed by those containing more than 20% water.

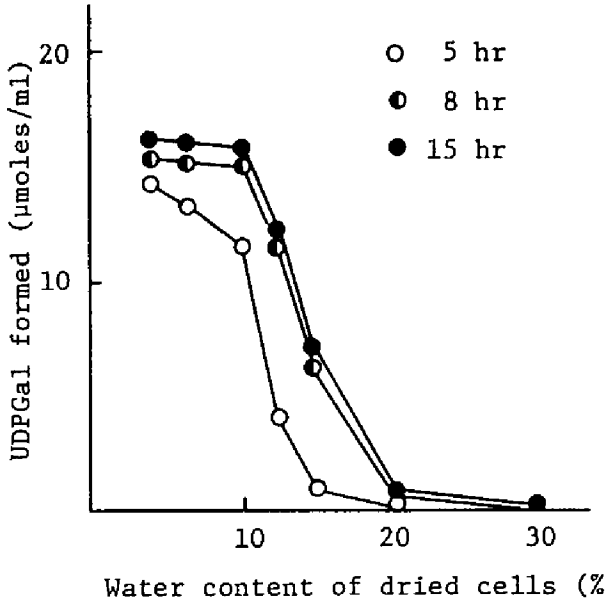


Fig. 1. Effect of Water Content of Lactose-Grown Dried Cells on Activity of UDPGal Formation.

The reaction system was described in Table I in which galactose was incubated as sugar substrate.

Similar experiment was done with dried cells of *T. candida* which was grown on glucose instead of lactose. As is shown in Fig. 2-(A), it was observed that the activity of UDPG formation by the glucose-grown cells was also affected by changing degree of desiccation of dried cells, but the activity was less sensitive to their water content than that in the lactose-grown cells as shown in Fig. 2-(B).

Effects of sugar substrates and water content of lactose-grown cells on UDP-hexose forming activities

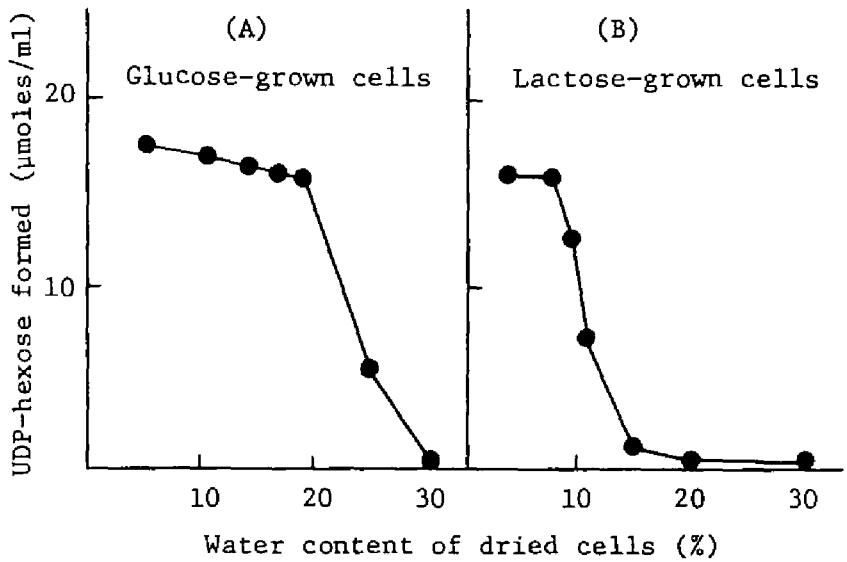


Fig. 2. Effect of Water Content of Glucose-Grown and Lactose-Grown Cells on Activity of UDP-Hexose Formation.

The reaction system was described in Table I in which glucose was incubated in (A), and galactose in (B), respectively. The reaction time was 8 hr.

From the results indicated above, it was clear that the activity of UDP-hexose formation by *T. candida* was markedly influenced by the degree of desiccation of the dried cells preparation to be used for the reaction. It was also shown that the activity of lactose-grown cells was more sensitive to their water content than that of glucose-grown cells. Then, the effect of sugar substrates on UDP-hexose fermentation by lactose-grown cells was investigated to clarify whether the difference of sensitivity to the water content of the cells was really due to the carbon sources for their growth medium.

As is shown in Fig. 3, little difference of UDP-hexose forming activity was observed between glucose and galactose used as sugar substrate irrespective of the water content of the dried cells.

It is suggested from the above results that the carbon sources of growth media for the yeast might somewhat affect the structure of the cell wall and that the permeability of reaction substrates into the cells and the extracellular excretion of the enzymes responsible to UDP-hexose fermentation might be changed by the water content of the dried yeast preparations. Moreover, the degree of autolysis during drying process of the cells may cause an inactivation of some of the related enzymes.

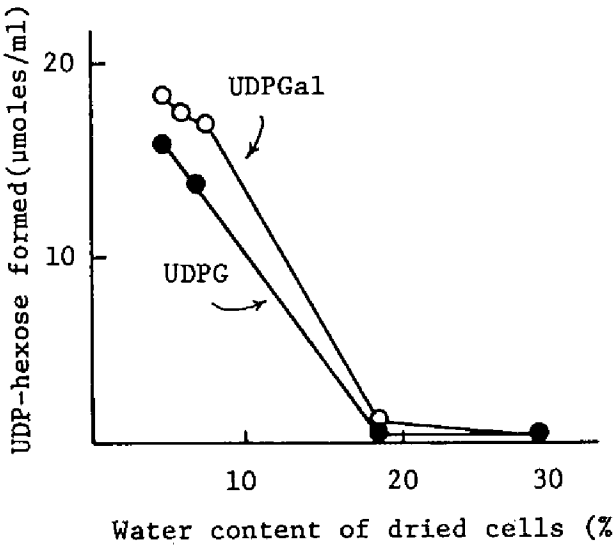


Fig. 3. Effect of Water Content of Lactose-Grown Dried Cells on Activity of UDP-Hexose Formation.

The reaction system was described in Table I in which lactose-grown cells were incubated with galactose (-O-) and glucose (-●-). The reaction time was 8 hr.

Then, the author examined the percentage of dead cells in glucose-grown and lactose-grown cells containing different amounts of water. The relationship between UDP-hexose forming activities and amounts of protein extracted from cells was investigated. As is shown in Table III, UDPG forming activity of the glucose-grown cells which contained 6.0% and 17.1% water was almost unchanged, but UDPGal was not formed by the lactose-grown cells when the water content increased from 7.8% to 18.1%. Further, it was observed that the amount of protein extracted from glucose-grown cells or lactose-grown cells was well parallel to the amount of UDP-hexose which was formed by the respective dried-cell preparations.

TABLE III. UDP-HEXOSE FORMING ACTIVITIES, AMOUNTS OF EXTRACTABLE PROTEIN AND PERCENTAGES OF DEAD CELLS IN GLUCOSE- AND LACTOSE-GROWN DRIED CELL PREPARATIONS

Cell preparations and their water content (%)	UDP-hexose formed* (μmoles/ml)		Protein extracted** (mg/ml)	Dead cells found (%)
	UDPG	UDPGal		
Glucose-grown cells (6.0)	18.5	-	3.73	88
Glucose-grown cells (17.1)	17.5	-	2.75	84
Lactose-grown cells (7.8)	-	18.5	2.55	55
Lactose-grown cells (18.1)	-	0	0.46	32

* The reaction time was 8 hr. The reaction system was described in Table I in which glucose was incubated with glucose-grown cells, and galactose was with lactose-grown cells.
** Five g of dried cells were suspended in 50 ml of 0.1M potassium phosphate buffer (pH 7.0), followed by shaking for 4 hr at 28°C. The supernatant solution was dialyzed overnight at 4°C.

Especially, little protein was extracted from the lactose-grown cells containing 18.1% water by which UDPGal was not formed. It is of interest that dead cells in larger percentages are found in the dried cells obtained from glucose medium than those from lactose medium, irrespective of their water contents. In other words, the lactose-grown cells had more resistance to desiccation than the glucose-grown cells from which the enzyme protein was easily extracted.

Water content of lactose-grown cells and enzyme activities in cell-free extract

Table IV shows the relationship between water content of the lactose-grown cells and some of their enzyme activities responsible to UDPGal synthesis. The total amounts of protein extracted from dried cells containing 5.9% and 17.2% water were 147 mg and 44.5 mg, respectively. UDPGal was not formed by the dried cells having 17.2% water, the result being in good agreement with that shown in Table III. However, an apparent increase in the amounts of enzyme protein and specific activities was observed when the water content decreased from 17.2% to 5.9%. About 2.5-fold increase was found in the specific activitiy of Gal-1-P uridylyltransferase which catalyzes the reaction: UDPG + Gal-1-P $\rightleftharpoons$ UDPGal + G-1-P.

From these results, it may be concluded that the effect of the water content of lactose-grown cells on UDPGal fermentation is caused by the degree of excretion of enzyme proteins which are related to

TABLE IV. ENZYME ACTIVITIES IN CELL-FREE EXTRACT OF LACTOSE-GROWN *T. candida*

Water content of dried cells	UDPG pyro-phosphorylase		Gal-1-P uridylyl-transferase		Galacto-kinase	
(%)	TU	SA	TU	SA	TU	SA
5.9	1528	10.4	948	6.45	846	5.76
17.2	270	6.07	113	2.54	197	4.44

TU: total units, SA: specific activity (units/mg protein)
The cell-free extract was prepared by the method described in Table III.

the synthesis of UDPGal. It is also suggested that the difference of UDP-hexose synthesizing activity between lactose-grown and glucose-grown cells may be due to the carbon sources of the growth media by which the structure and permeability of the cell wall may have been changed by the degree of desiccation of the yeast.

Lebedew[41] found that a glycolytic enzyme system could be easily extracted from dried yeast powder with warm water or buffer solution and that the fermentative activity of the Lebedew juice was weak unless dried cells underwent partial autolysis. As described in this work, the UDP-hexose forming activity of *T. candida* was remarkably influenced by the water content of dried-cell preparation, but this was overcome by the careful management of drying process of the cells. As is shown in Table V, it was possible to raise 5'-UMP

concentration up to 140 μmoles/ml in the reaction mixture and to convert 80% of the nucleotide to UDP-hexose (UDPGal 51.5 mg and UDPG 11.3 mg/ml) by the use of well-prepared dried cells.

TABLE V. EFFECT OF 5'-UMP CONCENTRATION ON UDP-HEXOSE FERMENTATION BY LACTOSE-GROWN *T. candida*

5'-UMP added (μmoles/ml)	Reaction time (hr)	Total UDP-hexose formed* (μmoles/ml)	UDPG formed** (μmoles/ml)	UDPGal formed (μmoles/ml)
	8	17.2	-	-
20	16	17.5	-	-
	24	17.1	3.3	13.7
	8	35.4	-	-
60	16	54.4	-	-
	24	51.0	7.8	43.0
	8	46.5	-	-
100	16	86.8	-	-
	24	84.7	10.4	74.2
	8	28.5	-	-
140	16	51.6	-	-
	24	112.1	20.2	91.7

The reaction system was described in Table I in which galactose was incubated as sugar substrate, and the concentration of 5'-UMP was varied as shown above.

* determined by paper chromatography

** determined by enzymic assay

SUMMARY

The UDP-hexose fermentation by *Torulopsis candida* was remarkably influenced by the preparative conditions of dried cells used as enzyme source. It was necessary to use the lactose-grown cells which had less than 10% of water for a maximal formation of UDPGal. Little accumulation of UDPGal occurred when the water content of dried cells increased to more than 20%. In case of glucose-grown cells, the activity of UDPG formation was less sensitive to their water content than that of UDPGal formation observed in the lactose-grown cells.

The amounts of extractable protein from glucose-grown and lactose-grown cells of the yeast were well parallel to the amounts of UDPG and UDPGal which were formed by the respective dried cells. By lowering the water content of lactose-grown cells from 17.2% to 5.9%, an apparent increase occurred in the amounts of enzyme protein and enzyme activities responsible to UDPGal synthesis. It was concluded from these results that the effect of the water content of lactose-grown cells on UDPGal fermentation was due to the degree of excretion of enzyme proteins which were related to the synthesis of UDPGal.

Section 3. Mechanism of UDP-Galactose Fermentation

INTRODUCTION

The biological importance of UDPGal and UDPG has now been completely established on the Leloir pathway for galactose metabolism.[25] The biosynthetic route of these uridine coenzymes has a close relation to galactose metabolism in many organisms and can be summarized as follows:

$$\text{Galactose} + \text{ATP} \xrightarrow{\text{Galactokinase}} \text{Gal-1-P} + \text{ADP} \qquad (1)$$

$$\text{Gal-1-P} + \text{UDPG} \underset{\text{Gal-1-P uridylyl-transferase}}{\rightleftharpoons} \text{UDPGal} + \text{G-1-P} \qquad (2)$$

$$\text{UDPGal} \underset{\text{UDPGal 4-epimerase}}{\rightleftharpoons} \text{UDPG} \qquad (3)$$

The formation of catalytic amount of UDPG needed for the initiation of the reaction (2) is supplied by a forth reaction:

$$\text{G-1-P} + \text{UTP} \underset{\text{UDPG pyrophosphorylase}}{\rightleftharpoons} \text{UDPG} + \text{PPi} \qquad (4)$$

Another pathway leading to the direct formation of UDPGal is as follows:

$$\text{Gal-1-P} + \text{UTP} \underset{\text{UDPGal pyrophosphorylase}}{\rightleftharpoons} \text{UDPGal} + \text{PPi} \qquad (5)$$

In the previous sections, it was described that a remarkable amount of UDPGal was formed and accumulated by air-dried cells of lactose-grown *Torulopsis candida* IFO 0768 when incubated aerobically with 5'-UMP and galactose or lactose in the presence of high concentration of inorganic phosphate. It was also found that UDPG was produced in high yields under the same reaction conditions if galactose or lactose was replaced by glucose.

It is of primary interest to try to find out whether UDPGal formation from 5'-UMP proceeds along the Leloir pathway and why it accumulates efficiently under the reaction conditions using air-dried cells as enzyme sources. The present work was performed to elucidate the mechanism of UDPGal fermentation by the yeast.

MATERIALS AND METHODS

Materials. Gal-1-P was purchased from Boehringer and Soehne GmbH, Mannheim. All other chemicals were the same as used in the previous sections.

Microorganism and cultivation. *Torulopsis candida* IFO 0768 was used throughout this work. The yeast was cultivated at 28°C for 24 to 48 hr on a medium containing 5% lactose as carbon source as described in the previous section. The cells harvested by centrifugation were washed three times with tap water, air-dried at room temperature for 24-36 hr, and desiccated overnight under reduced

pressure over P_2O_5. The dried cells were kept at -20°C until used.

Enzyme preparations. UDPG dehydrogenase was prepared as described in the previous section. The cell-free extract and ammonium sulfate fraction of *T. candida* were prepared as follows. To 10 g of the dried cells, were added 20 g of alumina and 15 ml of 0.1 M potassium phosphate buffer (pH 7.2), and ground in a cooling mortar for 30 min. Then, it was suspended into 40 ml of the same buffer and subjected to sonication with a 20 Kc Kaijo Denki oscillator at 0-10°C for 15 min. The debris was removed by centrifugation at 12,000 x g at 0°C for 30 min. The supernatant solution which was dialyzed overnight against 0.01 M potassium phosphate buffer (pH 7.2) containing 0.05% 2-mercaptoethanol was employed as cell-free extract. The cell-free extract was brought to 30% saturation with solid ammonium sulfate and the precipitate was removed by centrifugation. The precipitate obtained by addition of ammonium sulfate to 80% was dissolved in 0.01 M potassium phosphate buffer (pH 7.2) containing 0.05% 2-mercaptoethanol and dialyzed against the same buffer.

Analytical method. UDPG and UDPGal were determined by the methods described previously. Protein was estimated by the method of Lowry *et al.*[40] Enzyme reactions with cell-free extract and ammonium sulfate fraction were terminated by immersing the tubes in boiling water for 1 min. The reactions with dried cells as enzyme sources were terminated by immersing the tubes in boiling water for 2 min.

Each aliquot of the supernatant solutions was subjected to analyses. Galactose was estimated by the method of Somogyi[36] after treating the reaction mixture with 5% $ZnSO_4$ and 0.3 N $Ba(OH)_2$. Enzyme activities were estimated under the conditions as described in figures and tables.

RESULTS AND DISCUSSION

Enzyme activities involved in galactose metabolism

The activation of galactose is initiated through a direct phosphorylation at the reducing group, giving rise to Gal-1-P as shown in the reaction (1). The enzyme, galactokinase, was first found by Leloir and coworkers[42] in *Saccharomyces fragilis* adapted to galactose. Table I shows the presence of the enzyme activity in cell-free extract of *T. candida* grown on a lactose medium. Rapid consumption of galactose was observed only in the presence of ATP. Fluoride was effective to protect ATP and Gal-1-P from being dephosphorylated by phosphatase.

The enzyme, Gal-1-P uridylyltransferase,[38] catalyzes the incorporation of Gal-1-P into UDPG to form UDPGal and G-1-P (reaction 2), the latter being metabolized *via* glycolytic pathway. As is shown in Table II, UDPG was consumed by cell-free extract when incubated with galactose and ATP. The amount of UDPGal formed was almost equal to that of UDPG consumed. No formation of UDPGal

TABLE I. GALACTOKINASE ACTIVITY IN CELL-FREE EXTRACT OF LACTOSE-GROWN *T. candida*

Reaction system	Galactose consumed (µmoles)	
	30 min	60 min
Complete system	2.70	3.30
" -ATP, -NaF	0	0
" -NaF	1.87	1.87

The complete system contained 5 µmoles of galactose, 10 µmoles of ATP, 60 µmoles of NaF, 10 µmoles of $MgCl_2$, 250 µmoles of Tris-HCl buffer (pH 7.5) and 10 mg protein of cell-free extract in a final volume of 3 ml. Incubation was carried out at 30°C for 30 and 60 min.

TABLE II. GAL-1-P URIDYLYLTRANSFERASE ACTIVITY IN CELL-FREE EXTRACT OF LACTOSE-GROWN *T. candida*

Reaction system	UDPG consumed (µmoles)	UDPGal formed (µmoles)
Complete system	6.4	6.2
" -ATP	0.4	0
" -ATP,-galactose	0.3	0

The complete system contained 8 µmoles of UDPG, 10 µmoles of galactose, 10 µmoles of ATP, 10 µmoles of $MgCl_2$, 400 µmoles of glycine buffer (pH 8.8) and 5.3 mg protein of cell-free extract in a final volume of 2.5 ml. Incubation was carried out at 30°C for 60 min.

occurred unless galactose or ATP was present. The results would strongly suggest the presence of Gal-1-P uridylyltransferase in the cell-free extract.

UDPG pyrophosphorylase catalyzes the transfer of uridylyl group from UTP to G-1-P with the formation of UDPG and inorganic pyrophosphate as shown in the reaction (4). The enzyme was first found in non-adapted yeast[43] and is abundant in many organisms. Analogous reaction catalyzed by UDPGal pyrophosphorylase proceeds according to the reaction (5) to form UDPGal and inorganic pyrophosphate, the enzyme being considered to play a dominant role in plant tissues,[44] though it was first found in small amounts in galactose-adapted yeast and mammalian liver.[45] Both pyrophosphorylase activities toward sugar nucleotide formation were investigated with ammonium sulfate fraction of *T. candida*. As is shown in Table III, UTP was consumed rapidly with the formation of UDPG when incubated with G-1-P, but not with Gal-1-P. This data would indicate the presence of high UDPG pyrophosphorylase activity and the absence of UDPGal pyrophosphorylase. It was also confirmed by examining the reverse reaction, that is, pyrophosphorolysis of UDPG or UDPGal with the same enzyme preparation. Then, appreciable amounts of UTP were formed in UDPGal-PPi system, though the rate of its formation was smaller than that in UDPG-PPi system. Therefore, UDPGal pyrophosphorylase activity of the yeast can not be neglected from these

TABLE III. UDPG PYROPHOSPHORYLASE AND UDPGAL PYROPHOSPHORYLASE ACTIVITIES IN AMMONIUM SULFATE FRACTION OF LACTOSE-GROWN *T. candida*

Incubation time (min)	UDPG pyrophosphorylase activity		UDPGal pyrophosphorylase activity	
	UTP consumed (μmoles)	UDPG formed (μmoles)	UTP consumed (μmoles)	UDPGal formed (μmoles)
10	5.2	6.2	0	0
20	9.1	9.4	0	0
30	9.5	9.9	0	0
60	9.6	10.0	trace	trace
90	9.7	9.5	trace	trace

The reaction mixture for UDPG pyrophosphorylase contained 10 μmoles of G-1-P, 17.7 μmoles of UTP, 5 μmoles of $MgCl_2$, 300 μmoles of glycine buffer (pH 8.8) and 2.5 mg protein of ammonium sulfate fraction in a final volume of 1.7 ml. The reaction mixture for UDPGal pyrophosphorylase was the same as indicated above except that G-1-P was replaced by Gal-1-P. Incubation was carried out at 30°C.

results, the details being under investigation. It is also suggested from Table III that significant amounts of inorganic pyrophosphatase may be contaminated in the enzyme preparation to push the reaction toward UDPG formation. Experiment was performed to prove this point and the strong activity was demonstrated as shown in Table IV.

TABLE IV. INORGANIC PYROPHOSPHATASE ACTIVITY IN CELL-FREE EXTRACT OF LACTOSE-GROWN *T. candida*

Incubation time (min)	Pi formed (μmoles)
20	16.2
40	17.1

The reaction mixture contained 20 μmoles of sodium pyrophosphate, 20 μmoles of $MgCl_2$, 200 μmoles of veronal buffer (pH 7.2), and 7.3 mg protein of cell-free extract in a final volume of 3 ml. As the enzyme solution contained small amount of inorganic phosphate, control incubation was carried out with enzyme boiled at 100°C for 2 min. Incubation was carried out at 30°C. Inorganic phosphate was determined by the method of Fiske-Subbarow.[73]

Effects of UDPG and G-1-P on formation of UDPGal by cell-free system

From the results indicated above, *T. candida* grown on lactose was found to have enzyme systems related to galactose pathway. Then, the experiments were performed with the cell-free extract to confirm whether UDPGal formation proceeds in combination with the reaction (2) and the reaction (4) where UDPG or G-1-P is needed as a catalyst. To the reaction mixture composed of excess Gal-1-P and UTP, was added either UDPG or G-1-P in various amounts with the cell-free extract. The results are given in Fig. 1 and Fig. 2.

As can be seen in Fig. 1-(a), the addition of UDPG affected the rate of UDPGal formation remarkably. Although appreciable amounts of UDPGal were formed gradually even in the absence of UDPG, an apparent increase was observed in the initial rate of UDPGal formation by adding a catalytic amount of UDPG of 0.5-5 x 10^{-3} M. Moreover, the amount of UDPGal formed came to exceed that of UDPG added with incubation time.

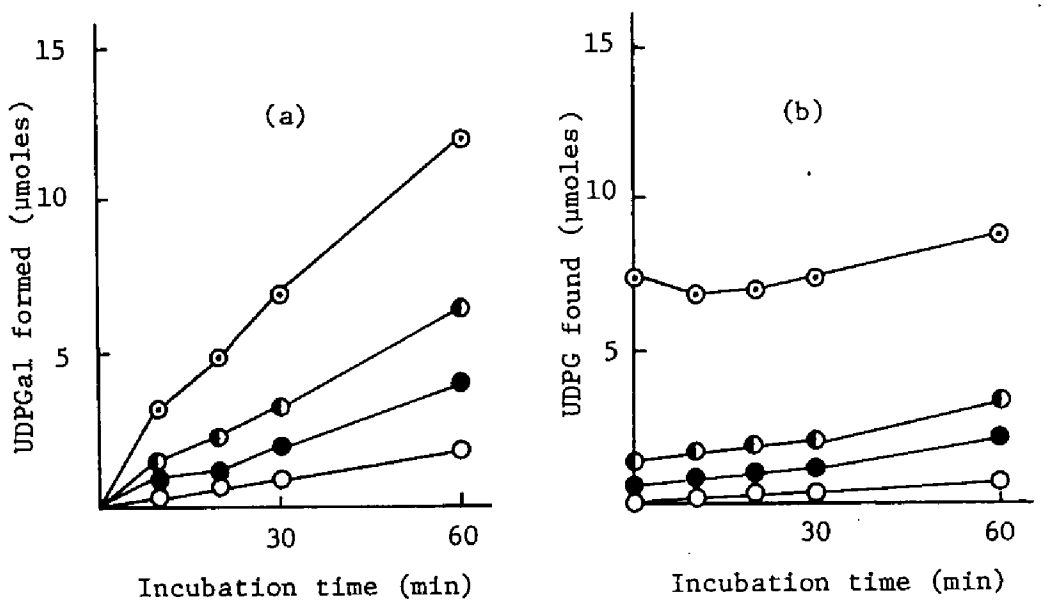


Fig. 1. Effect of UDPG on Formation of UDPGal from Gal-1-P and UTP by Cell-Free Extract of *T. candida*.

The reaction mixture contained 20 μmoles of Gal-1-P, 30 μmoles of UTP, 300 μmoles of potassium phosphate buffer (pH 7.2), 10 μmoles of $MgCl_2$, 10.7 mg protein of cell-free extract and indicated amounts of UDPG in a final volume of 1.5 ml.
UDPG added; —○— 0, —●— 5 x 10^{-4}M, —◐— 1 x 10^{-3}M, —⊙— 5 x 10^{-3}M

The results indicate that UDPGal formation proceeds mainly by the coupling reaction catalyzed by UDPG pyrophosphorylase and Gal-1-P uridylyltransferase. On the other hand, as shown in Fig. 1-(b), a gradual increase of UDPG is observed even without initial addition of UDPG, which may probably be derived from G-1-P contaminated in the cell-free extract by means of UDPG pyrophosphorylase. This observation leads to the suggestion that a small amount of UDPGal formed in the absence of UDPG is not entirely due to UDPGal pyrophosphorylase but to the coupling reaction with UDPG pyrophosphorylase and Gal-1-P uridylyltransferase.

The effect of G-1-P is shown in Fig. 2. By the addition of catalytic amounts of G-1-P to UTP-Gal-1-P system, the rate of UDPGal formation was clearly accelerated as compared to that in the absence of the sugar phosphate as shown in Fig. 2-(a). On the other hand, Fig. 2-(b) indicated that in the early stage of incubation relatively higher amounts of UDPG accumulated with addition of G-1-P prior to the formation of UDPGal, that is, the rate of UDPG formation in the presence of G-1-P was higher than that of UDPGal formation observed in the early incubations. Moreover, when the concentration of UDPG attained to a certain lebel, it began to decrease again to be used for UDPGal synthesis.

All the results indicated in Figs. 1 and 2 strongly suggest that UDPGal formation by cell-free extract of *T. candida* may proceeds

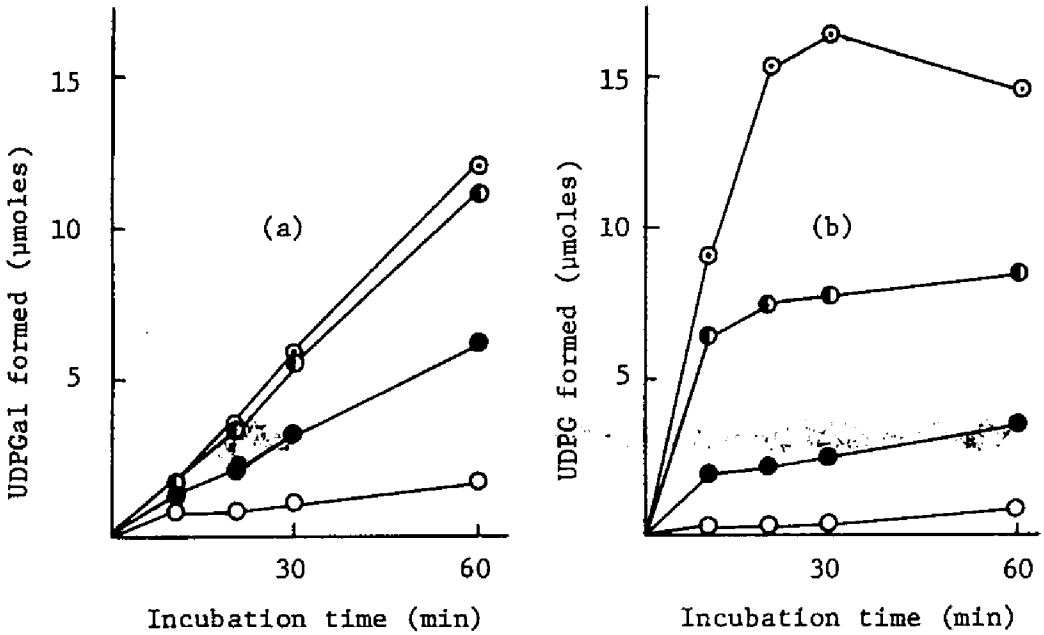


Fig. 2. Effect of G-1-P on Formation of UDPGal from Gal-1-P and UTP by Cell-Free Extract of *T. candida*.

The reaction mixture contained 20 μmoles of Gal-1-P, 30 μmoles of UTP, 300 μmoles of potassium phosphate buffer (pH 7.2), 10 μmoles of $MgCl_2$, 10.7 mg protein of cell-free extract and indicated amounts of G-1-P in a final volume of 1.5 ml. G-1-P added; —○— 0, —●— 1 x 10^{-3}M, —◐— 5 x 10^{-3}M, —⊙— 1 x 10^{-2}M

by a coupling reaction catalyzed by UDPG pyrophosphorylase and Gal-1-P uridylyltransferase where a catalytic amount of UDPG or G-1-P is needed with a participation of inorganic pyrophosphatase to rotate the reaction smoothly.

UDPGal fermentation and inhibition of UDPGal 4-epimerase

A typical time course of UDPGal fermentation from 5'-UMP and

galactose by dried cells of lactose-grown *T. candida* is shown in Fig. 3. As was already described in the section 1, 5'-UMP was rapidly decreased, followed by the accumulation of UTP, UDP and UDPG in the early period of fermentation. Subsequently, UDPGal began to appear gradually and reached a maximum with disappearance of UTP and UDP.

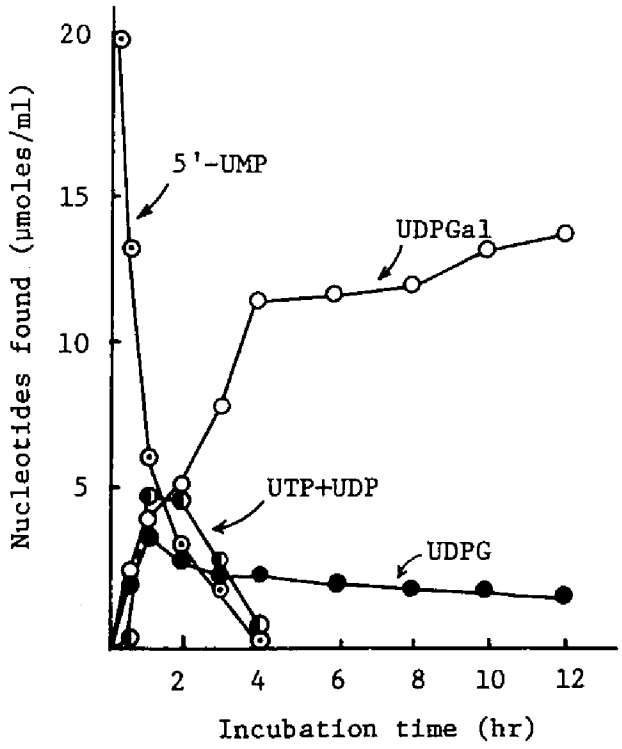


Fig. 3. Fermentative Production of UDPGal from 5'-UMP and Galactose by Dried Cells of *T. candida*.

The reaction mixture contained 50 μmoles of 5'-UMP, 500 μmoles of galactose, 500 μmoles of potassium phosphate buffer (pH 7.4), 25 μmoles of $MgCl_2$ and 250 mg of dried cells in a final volume of 2.5 ml. The reaction was carried out at 30°C by shaking.

The final yield of UDPGal accumulated was about 70% based on initial 5'-UMP added. At the same time a small amount of UDPG (less than 10% of UDPGal) was found to accumulate, though it tended to decrease with prolonged incubation. It is a striking characteristic of this fermentation that UDPGal once accumulated is neither decreased nor degraded under the reaction condition. If UDPGal 4-epimerase (reaction 3) is actually operating under the fermentative condition, the amount of UDPG formed should exceed that of UDPGal after equilibrium is reached, since the epimerase reaction lies so far toward UDPG, the equilibrium being attained with 75% UDPG and 25% UDPGal in *S. fragilis*.[4] But this was not the case for UDPGal fermentation by dried cells of *T. candida* as mentioned above. Therefore, it can be assumed that UDPGal 4-epimerase activity of the yeast must be very low or repressed for some reasons under the reaction condition.

It has recently been reported[46,47] that 5'-UMP and some specific sugars such as D-galactose, D-fucose and L-arabinose which relate to induction and repression of the biosynthesis of UDPGal 4-epimerase in *S. fragilis*, exerted a concerted action on UDPGal 4-epimerase fluorescence, accompanied by a reductive inactivation of the enzyme. It is also reported[48] that the catalytic activity of the reduced epimerase of *S. fragilis* caused by a combination with 5'-UMP and galactose is much lower than that of native epimerase.

Then, an experiment was done to check the epimerase activity

of *T. candida* and to elucidate whether the activity was affected by 5'-UMP and galactose under the fermentative condition using dried cells. As is shown in Fig. 4, about 65% of UDPGal is converted to UDPG in a relatively short incubation time (control system), which strongly indicates the presence of UDPGal 4-epimerase in the yeast.

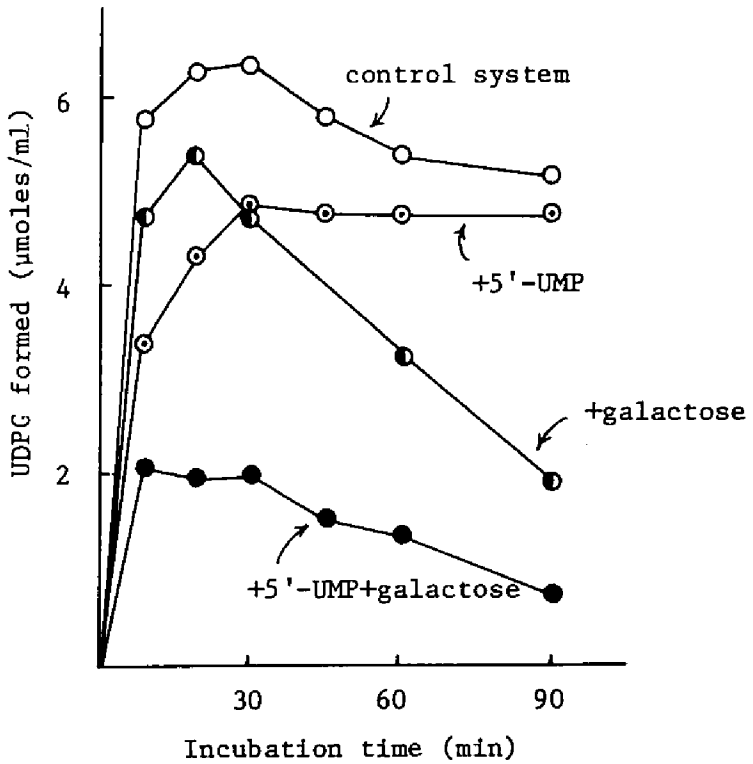


Fig. 4. Effect of 5'-UMP and Galactose on Conversion of UDPGal to UDPG by Dried Cells of *T. candida*.

The reaction system (control) contained 9.7 μmoles of UDPGal, 200 μmoles of potassium phosphate buffer (pH 7.0) and 100 mg of dried cells in a final volume of 1 ml. To the control, was added either 5'-UMP (20 μmoles/ml) or galactose (200 μmoles/ml), or in combination. The reaction was carried out at 30°C by shaking.

It was also found that UDPG once accumulated in the galactose containing system was rapidly consumed again. This may probably be due to the reaction (1) catalyzed by galactokinase and the reaction (2) catalyzed by Gal-1-P uridylyltransferase which were demonstrated in cell-free extract of the yeast as described before. However, the conversion of UDPGal to UDPG was strikingly repressed by incubation with 5'-UMP provided that galactose was also present. Therefore, it is suggested that the epimerase of the yeast may be unable to operate under the condition of UDPGal fermentation where both 5'-UMP and galactose are present. In other words, the coexistence of 5'-UMP and galactose added as substrates for UDPGal production serves efficiently to protect the product UDPGal against its reconversion to UDPG. The details on the nature of a partially purified *Torulopsis* epimerase with respect to inhibition by 5'-UMP and sugars will be described in the next section.

The concerted effect of 5'-UMP and galactose on the conversion of UDPGal to UDPG by the dried cells was investigated with respect to their concentration. Figure 5 shows the effect of 5'-UMP concentration in the absence or presence of 2×10^{-1} M galactose. The initial rates of the conversion of UDPGal to UDPG were obviously decreased by the addition of increasing amounts of 5'-UMP. A maximum inhibition of the conversion was attained with the concentrations of 2×10^{-1} M galactose and 2×10^{-2} M 5'-UMP. The rates of disappearance

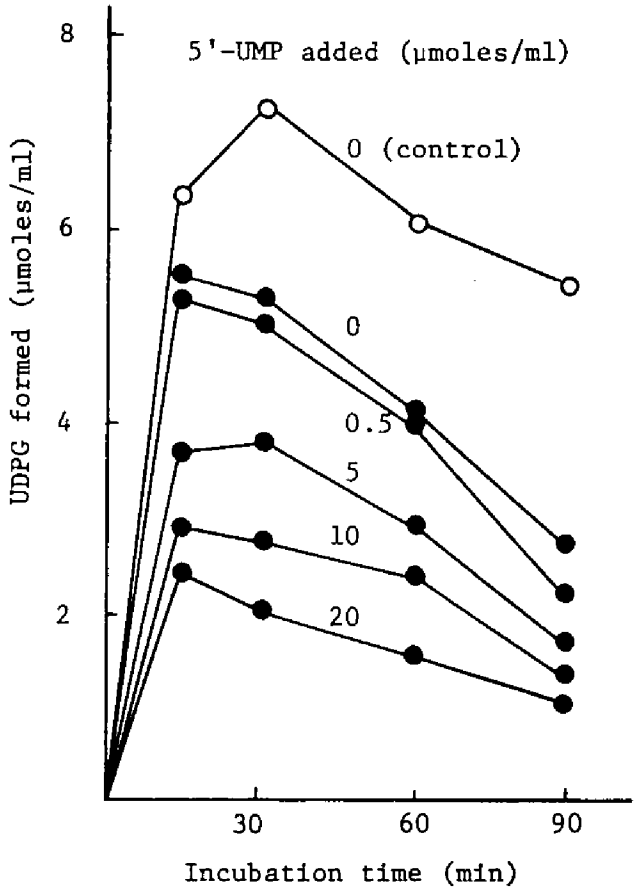


Fig. 5. Effect of 5'-UMP Concentration on Conversion of UDPGal to UDPG by Dried Cells of *T. candida*.

The reaction mixture contained 9.7 μmoles of UDPGal, 200 μmoles of potassium phosphate buffer (pH 7.0), 200 μmoles of galactose, 100 mg of dried cells and indicated amounts of 5'-UMP in a final volume of 1 ml. Galactose was omitted in the control (—○—). The reaction was carried out at 30°C with shaking.

of UDPG became faster with decreasing concentration of 5'-UMP.

The effect of galactose concentration is shown in Fig. 6. The rates of UDPG formation also decreased with increasing concentration of galactose, a minimum rate being attained at the concentration of

2×10^{-1} M galactose and 2×10^{-2} M 5'-UMP. It seems from these results that the concentration of 5'-UMP present is more critical than that of galactose for the conversion of UDPGal to UDPG in the later stage of the reaction. In other words, the conversion is to be inhibited by the presence of a relatively low concentration of 5'-UMP, provided that enough galactose is present.

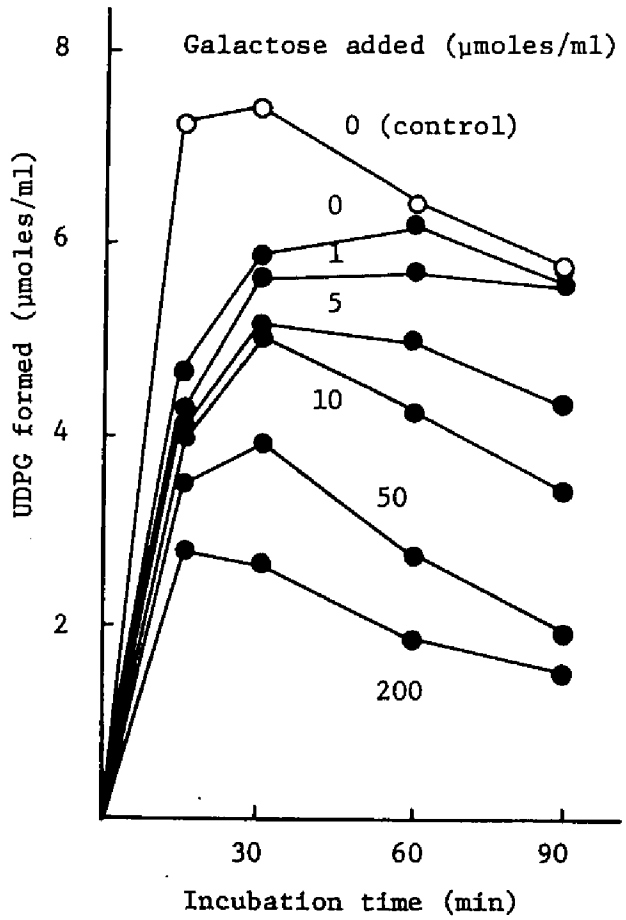


Fig. 6. Effect of Galactose Concentration on Conversion of UDPGal to UDPG by Dried Cells of *T. candida*.

The reaction mixture contained 9.7 μmoles of UDPGal, 200 μmoles of potassium phosphate buffer (pH 7.0), 20 μmoles of 5'-UMP, 100

mg of dried cells and indicated amounts of galactose in a final volume of 1 ml. 5'-UMP was omitted in the control (—○—). The reaction was carried out at 30°C by shaking.

From the data described so far, it is concluded that the fermentative production of UDPGal from 5'-UMP and galactose by dried cells of *T. candida* will proceed by the coupling reactions catalyzed by UDPG pyrophosphorylase and Gal-1-P uridylyltransferase. The mechanism of UDPGal accumulation might also be explained by a characteristic nature of UDPGal 4-epimerase of the yeast which undergoes a concerted inhibition by the substrates of the fermentation, 5'-UMP and galactose. The mechanism of UDPGal accumulation under the fermentative condition is illustrated in Fig. 7. As is already described, 5'-UMP is easily phosphorylated to form UTP in the early stage of the fermentation by nucleoside monophosphate kinase and nucleoside diphosphate kinase. G-1-P may probably be derived from glycogen involved in the dried cells. In fact, UDPG accumulates when fermentation is carried out without addition of energy sources, but this is not the case when the dried cells are preliminarily allowed to auto-respiration for several hours before they are used as enzyme sources (unpublished observation). If glucose is supplied together with galactose as energy source, G-1-P may generate more easily *via* G-6-P by the catalysis of hexokinase and phosphoglucomutase. On the other hand, Gal-1-P is accumulating from galactose or lactose by the

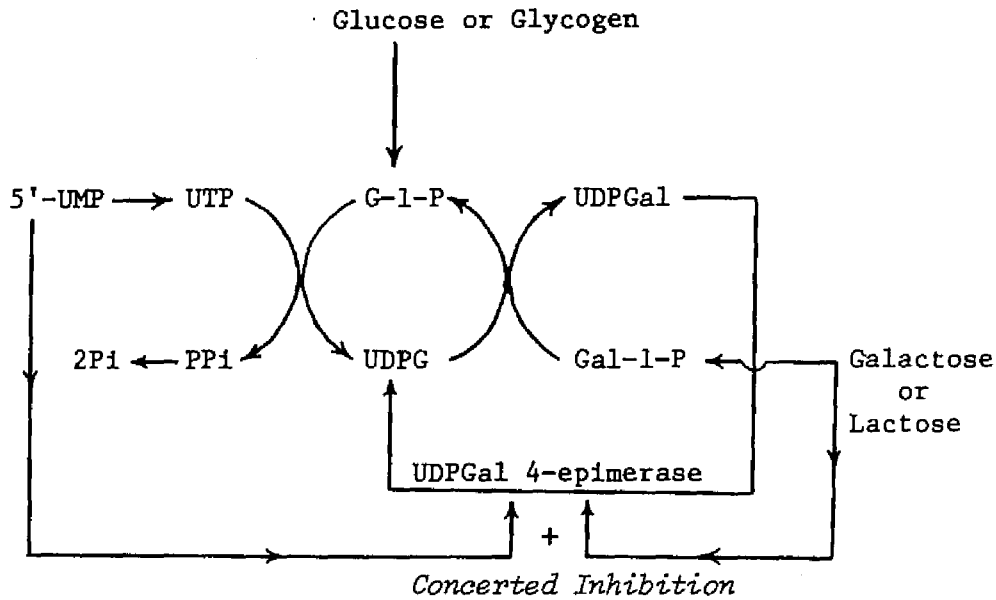


Fig. 7. Mechanism of UDPGal Fermentation by *T. candida*.

catalysis of galactokinase to be converted to UDPGal. Thus, Gal-1-P is incorporated into UDPG to form UDPGal and G-1-P by Gal-1-P uridylyltransferase, the latter sugar phosphate being utilized again to form UDPG by UDPG pyrophosphorylase. Further, the action of inorganic pyrophosphatase is also essential to push the pyrophosphorylase reaction toward UDPG formation. It is a key point of this UDPGal fermentation that there occurs a concerted inhibition by 5'-UMP and galactose on conversion of UDPGal to UDPG under the fermentative condition. The enzymic characteristics of UDPGal 4-epimerase of *T. candida* will be presented in the next section.

SUMMARY

Enzyme activities involved in the galactose metabolism of *T. candida* grown on a lactose medium were investigated with the cell-free extract and ammonium sulfate fraction. Remarkable activities of galactokinase, Gal-1-P uridylyltransferase and UDPG pyrophosphorylase were detected, whereas UDPGal pyrophosphorylase activity was weak. UDPGal formation proceeded by the cell-free extract along a coupling reaction catalyzed by UDPG pyrophosphorylase and Gal-1-P uridylyltransferase where UDPG or G-1-P acted as a catalyst.

The mechanism of UDPGal accumulation under the fermentative condition could be explained by a concerted inhibition of UDPGal 4-epimerase activity by 5'-UMP and galactose present as fermentation substrates.

Section 4. NAD-Dependent UDP-Galactose 4-Epimerase and Its Inactivation by 5'-UMP and Galactose

INTRODUCTION

Uridine diphosphate galactose 4-epimerase (UDPGal 4-epimerase EC 5.1.3.2) which catalyzes the reversible conversion of UDPGal into UDPG was first found by Leloir in galactose-adapted yeast.[4] The enzyme is found in mammalian systems, yeasts, bacteria and plants.[49] It has been partially purified from calf liver acetone powder,[26] and highly purified from galactose-adapted *Saccharomyces fragilis*,[30,50,51] *Escherichia coli*,[52-54] bovine mammary gland,[55] and wheat germ.[56] It has also been reported that the calf liver enzyme has an absolute requirement for NAD,[26] while neither the *S. fragilis* enzyme[30,50] nor the *E. coli* enzyme[53] requires exogenous NAD, since the coenzyme binds tightly to the enzyme protein.

On the other hand, Kalckar *et al.*[46-48,57,58] have recently reported that 5'-UMP and specific sugars such as D-galactose, D-fucose and L-arabinose which relate to induction and repression of the biosynthesis of UDPGal 4-epimerase, brought about an enhancement of UDPGal 4-epimerase fluorescence, accompanied by an reductive inactivation of the enzyme obtained from *S. fragilis* and *E. coli*.

In the previous section, it was found that the conversion of UDPGal to UDPG by dried cells of *Torulopsis candida* was remarkably

inhibited by 5'-UMP provided that galactose coexisted. This suggested that an inhibition of UDPGal 4-epimerase activity of the yeast took place by the combination of 5'-UMP and galactose. Further investigation on the epimerase showed that a partially purified UDPGal 4-epimerase of *T. candida* required exogenous NAD for the full activity unlike that of *S. fragilis* or *E. coli*.

The present section deals with the nature of the partially purified epimerase of *T. candida* with respect to its NAD dependence. The concerted effects of nucleotides and sugars on the enzyme activity were also investigated.

MATERIALS AND METHODS

Materials. All the chemicals used in this work were the same as used in the previous sections.

Preparation of enzyme. *Torulopsis candida* IFO 0768 was cultivated on a lactose medium and air-dried as described previously. UDPGal 4-epimerase of the yeast was partially purified as follows. Ten grams of the dried cells were mixed with 20 g of alumina and 15 ml of 0.1 M potassium phosphate buffer (pH 7.2), and ground in a cooling mortar for 30 min. It was suspended into 40 ml of the same buffer and was subjected to sonication with a 20 Kc Kaijo Denki oscillator at 0-10°C for 15 min. The cell debris was removed by centrifugation at 12,000 x g at 0°C for 30 min. The supernatant

solution was dialyzed overnight against 2 liters of 0.01 M potassium phosphate buffer (pH 7.2) containing 0.05% 2-mercaptoethanol. The cell-free extract (30 ml) was brought to 30% saturation with solid ammonium sulfate and the precipitate was removed by centrifugation. The precipitate obtained by addition of ammonium sulfate to 60 % was dissolved in 10 ml of 0.01 M potassium phosphate buffer (pH 7.2) containing 0.05% 2-mercaptoethanol and dialyzed overnight against 2 liters of the same buffer. The dialyzed solution was again brought to 30% saturation with the salt and the precipitate was discarded. The precipitate obtained by further addition of ammonium sulfate to 60% was dissolved in 2 ml of the same buffer and dialyzed overnight against 2 liters of the same buffer. This fraction was used as a partially purified UDPGal 4-epimerase throughout this work.

Assay of enzyme activity. UDPGal 4-epimerase activity was assayed by determining the amounts of UDPG formed from UDPGal under the reaction condition as described in figures and tables. The incubation was carried out at 30°C, and terminated by immersing the tube in boiling water for 1 min. An aliquot of the supernatant solution was estimated for UDPG by UDPG dehydrogenase as described previously.

RESULTS

NAD requirement for Torulopsis UDPGal 4-epimerase

In the preliminary experiment of UDPGal 4-epimerase of *T. candida*, it was found that the cell-free extract of the yeast had very weak activity of epimerization of UDPGal to UDPG without addition of NAD. However, the activity was obviously enhanced by the addition of exogenous NAD. It has already been reported that the calf liver epimerase has an absolute requirement for NAD,[26] whereas the enzymes obtained from *S. fragilis* [30,50] and *E. coli*[53] do not require NAD for the activity because NAD binds tightly to their enzyme protein. As is shown in Fig. 1, the epimerase activity in ammonium sulfate fraction of *T. candida* is highly enhanced by the addition of NAD at the concentration of 4.5×10^{-3} M, but little activity was observed without NAD. In the presence of NAD, the equilibrium was attained at about 70% UDPG and 30% UDPGal. The result would suggest that the epimerase of *T. candida*, unlike that of *S. fragilis* or *E. coli*, contains loosely-bound NAD to its apoenzyme. The *Torulopsis* enzyme seems somewhat like to that of calf liver and bovine mammary gland which depends on the addition of exogenous NAD for the full activity.

Figure 2 shows the effect of NAD concentration on enzyme activity. The *Km* for NAD calculated from these data according to the method of Lineweaver and Burk[59] is 1.4×10^{-4} M. NAD could not be replaced by NADP, and NADH did not inhibit the epimerase activity at the concentration of 5×10^{-3} M. As compared to the enzymes from

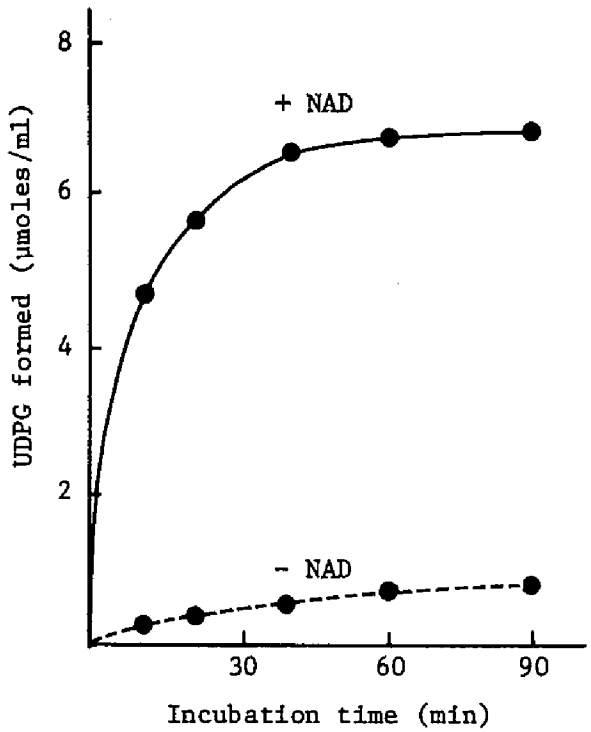


Fig. 1. NAD Requirement for UDPGal 4-Epimerase Activity. The reaction mixture contained 9.7 μmoles of UDPGal, 200 μmoles of glycine buffer (pH 8.8), 2.8 mg protein of enzyme and 4.5 μmoles of NAD in a final volume of 1 ml. Incubation was carried at 30°C.

calf liver and bovine mammary gland (*Km*s for NAD are 2×10^{-7} M and 5×10^{-7} M, respectively),[26,55] the *Torulopsis* enzyme showed relatively low affinity for NAD.

The effect of UDPGal concentration on enzyme activity is shown in Fig. 3. The *Km* for UDPGal calculated from these data is 1.2×10^{-3} M. The linear relationship between amounts of enzyme and UDPG

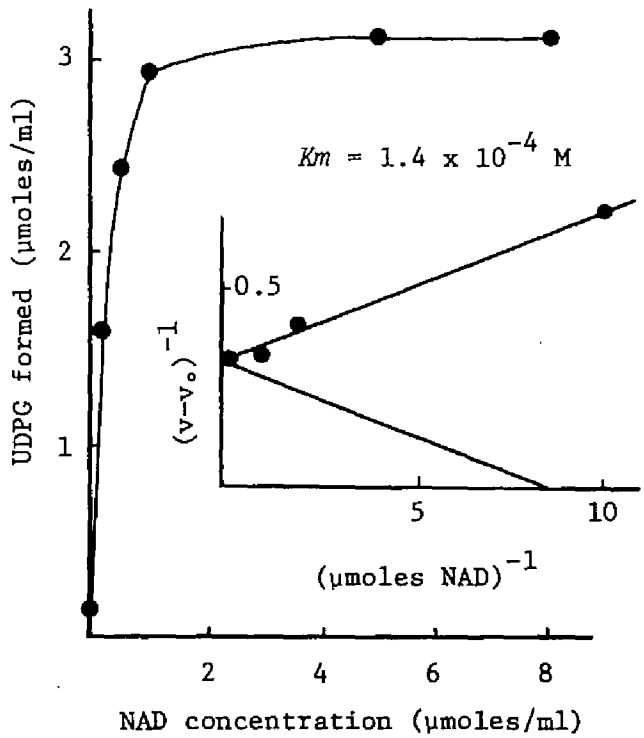


Fig. 2. Effect of NAD Concentration on UDPGal 4-Epimerase Activity.

The reaction mixture contained 9.7 μmoles of UDPGal, 200 μmoles of glycine buffer (pH 8.8), 1.4 mg protein of enzyme and indicated amounts of NAD in a final volume of 1 ml. Incubation was carried out at 30°C for 15 min.

formed during 10 min of incubation is shown in Fig. 4. The relationship between time of incubation and UDPG formed is shown in Fig. 5. UDPG was increased linearly for about 15 min incubation.

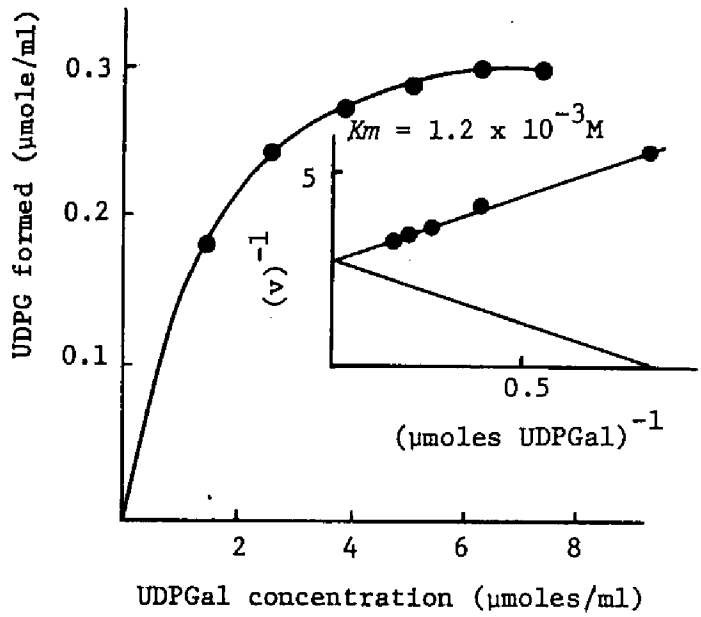


Fig. 3. Effect of UDPGal Concentration on UDPGal 4-Epimerase Activity.

The reaction mixture contained 5 μmoles of NAD, 200 μmoles of glycine buffer (pH 8.8), 240 μg protein of enzyme and indicated amounts of UDPGal in a final volume of 1 ml. Incubation was carried out at 30°C for 10 min.

Inhibition of UDPGal 4-epimerase by 5'-UMP and galactose

In the previous section, it was found that the conversion of UDPGal to UDPG by dried cells of *T. candida* was strikingly inhibited by the presence of 5'-UMP and galactose. This observation led to the suggestion that UDPGal 4-epimerase of the yeast might be inhibited by the combination of 5'-UMP and galactose, and consequently UDPGal could accumulate in large amounts without being degraded or converted to UDPG under the fermentation condition. It has recently been

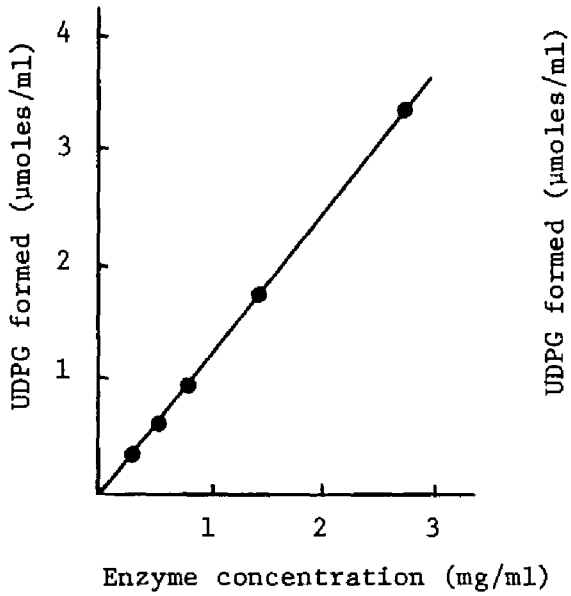


Fig. 4. Effect of Enzyme Concentration on UDPGal 4-epimerase Activity.

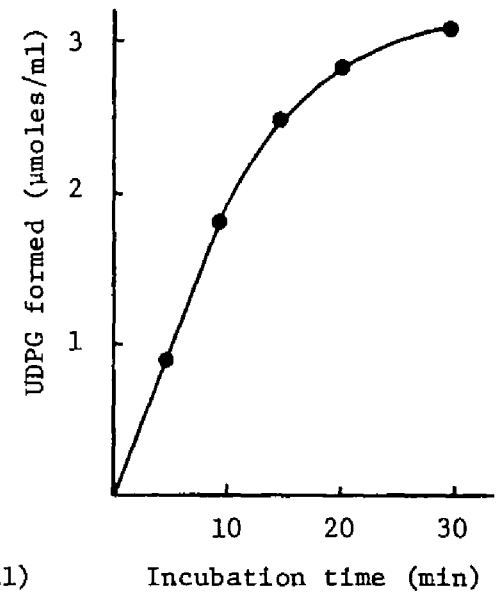


Fig. 5. Effect of Incubation time on UDPGal 4-Epimerase Activity.

The reaction mixture in Fig. 4 contained 9.7 μmoles of UDPGal, 5 μmoles of NAD, 200 μmoles of glycine buffer (pH 8.8) and indicated amounts of enzyme in a final volume of 1 ml. Incubation was carried out at 30°C for 10 min. The reaction mixture in Fig. 5 was the same as used in Fig. 4 except that 1.4 mg protein was employed.

reported by Kalckar *et al.*[47] that native epimerase preparations obtained from *S. fragilis* which are specific for NAD, are transformed into highly fluorescent reduced form (NADH) of epimerase by addition of 5'-UMP and galactose. They observed that the catalytic activity of the reduced epimerase was much lower than that of native epimerase (10-15% of native epimerase) and this reductive inactivation was also

found in crude epimerase preparations of *E. coli*, provided that 5'-UMP and galactose were present.[48] In view of these observations, the effect of 5'-UMP and galactose on the partially purified enzyme preparation of *T. candida* was investigated. As is shown in Fig. 6, the enzyme activity was remarkably inhibited by incubation with 5'-UMP, provided that galactose coexisted. Although slight inhibition of the activity occurred by addition of either galactose or 5'-UMP, the combination of the both brought about a maximum inhibition at the concentration of 1×10^{-2} M 5'-UMP and 1×10^{-1} M galactose.

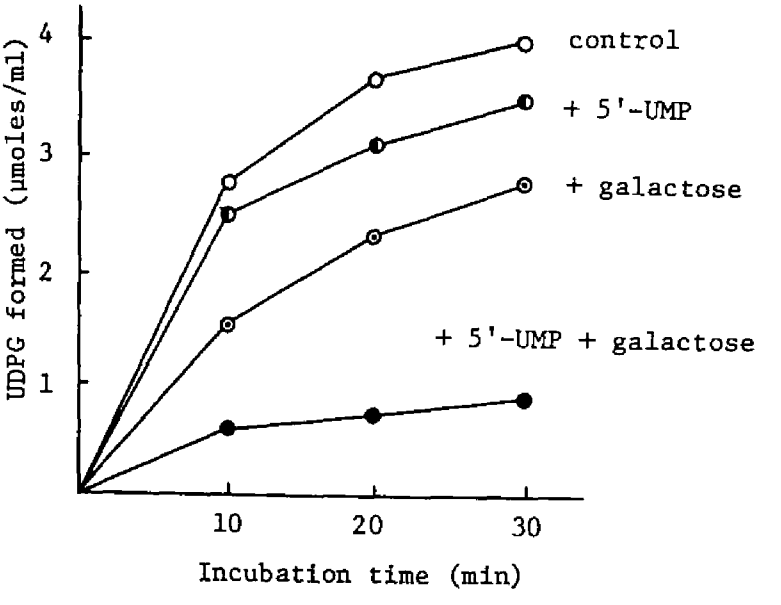


Fig. 6. Effect of 5'-UMP and Galactose on UDPGal 4-Epimerase.
The control mixture (—o—) contained 9.7 μmoles of UDPGal, 5 μmoles of NAD, 200 μmoles of gycine buffer (pH 8.8) and 1.4 mg protein of enzyme in a final volume of 1 ml. To the control, was added either 5'-UMP (10 μmoles/ml) or galactose (100 μmoles /ml), or in combination. They were preincubated with enzyme, NAD and buffer at 30°C for 10 min, followed by addition of UDPGal.

About 25% of activity in the control was found after 30 min incubation.

The effect of concentrations of 5'-UMP and galactose on enzyme activity is shown in Table I. It seems from these data that the concentration of 5'-UMP present is more critical than that of galactose for the inhibition.

TABLE I. EFFECT OF 5'-UMP AND GALACTOSE CONCENTRATIONS ON UDPGAL 4-EPIMERASE ACTIVITY

Additions (μmoles/ml)		Enzyme activity (%)
5'-UMP	Galactose	
0	0	100
10	0	79
10	1	71
10	5	61
10	10	53
10	50	27
0	100	86
0.5	100	35
1	100	31
5	100	25
10	100	24
15	100	24

The reaction mixture contained 9.7 μmoles of UDPGal, 5 μmoles of NAD, 200 μmoles of glycine buffer (pH 8.8), 1.55 mg protein and indicated amounts of 5'-UMP and galactose in a final volume of 1 ml. Either 5'-UMP or galactose, or in combination, was pre-incubated with enzyme, NAD and buffer at 30°C for 15 min, followed by the addition of UDPGal and incubated for 30 min.

Five μmoles/ml of 5'-UMP was enough to cause 75% inhibition, provided 100 μmoles/ml of galactose coexisted. Taking 5'-UMP concentration of 10 μmoles/ml as constant, more than 5 times galactose as much as 5'-UMP was necessary to cause 75% inhibition. That is to say, the inhibition occurs by the presence of a relatively low concentration of 5'-UMP provided that galactose is present enough. These data are also well consistent with those obtained in the previous section where the conversion of UDPGal to UDPG by dried cells of the yeast was extremely inhibited in the presence of 5'-UMP and galactose.

It is an interesting problem whether the epimerase inhibition by 5'-UMP and galactose is reversible or not. As shown in Table II, 85.5% of enzyme activity is recovered by a short dialysis of the enzyme preparations preincubated with 5'-UMP (2×10^{-2} M) and galactose (2×10^{-1} M), whereas only about 50% of activity is retained without dialysis. However, a strong inhibition was observed again by incubation with additional 5'-UMP and galactose to the preincubated dialyzed enzyme. These observations suggest that the inhibition of epimerase by 5'-UMP and galactose may probably be reversible and perhaps these inhibitors bind loosely to enzyme protein.

The inhibitory effect on enzyme activity was further investigated by using UDP, UTP and glucose other than 5'-UMP and galactose. As is shown in Fig. 7, a strong inhibition is also observed by incubating the enzyme with 5'-UMP, provided that glucose is present

TABLE II. REVERSIBILITY OF UDPGAL 4-EPIMERASE INHIBITION BY 5'-UMP AND GALACTOSE

Exp. No.	Treatment of enzyme preparations	Enzyme concentration (mg)	Enzyme activity (%)
(1)	Not preincubated with 5'-UMP + galactose	3.25	100
(2)	Preincubated with 5'-UMP + galactose at 30°C for 30 min	3.25	49.4
(3)	Preincubated with 5'-UMP + galactose at 30°C for 30 min and dialyzed	3.25	85.5
(4)	Preincubated with 5'-UMP + galactose at 30°C for 30 min and dialyzed. 5'-UMP and galactose were added again to the enzyme.	3.25	18.2

The reaction mixture contained 9.7 μmoles of UDPGal, 5 μmoles of NAD, 200 μmoles of glycine buffer (pH 8.8) and 3.25 mg protein of enzyme in a final volume of 1 ml. The incubation was carried out at 30°C for 30 min. In the Exp. (2), (3) and (4), the enzyme was preincubated with 5'-UMP (2×10^{-2} M) and galactose (2×10^{-1} M) at 30°C for 30 min. The dialysis of the preincubated enzyme was carried out at 4°C for 3 hr against 0.01 M potassium phosphate buffer (pH 7.2) containing 0.05% 2-mercaptoethanol. In the Exp. (4), 5'-UMP (20 μmoles) and galactose (200 μmoles) were added again to the dialyzed enzyme prior to the incubation.

instead of galactose. On the other hand, when 5'-UMP was replaced by UDP or UTP in combination with galactose or glucose, a slight inhibitory effect was observed, but it became to lower in the order of the combinations with UDP + hexoses and UTP + hexoses.

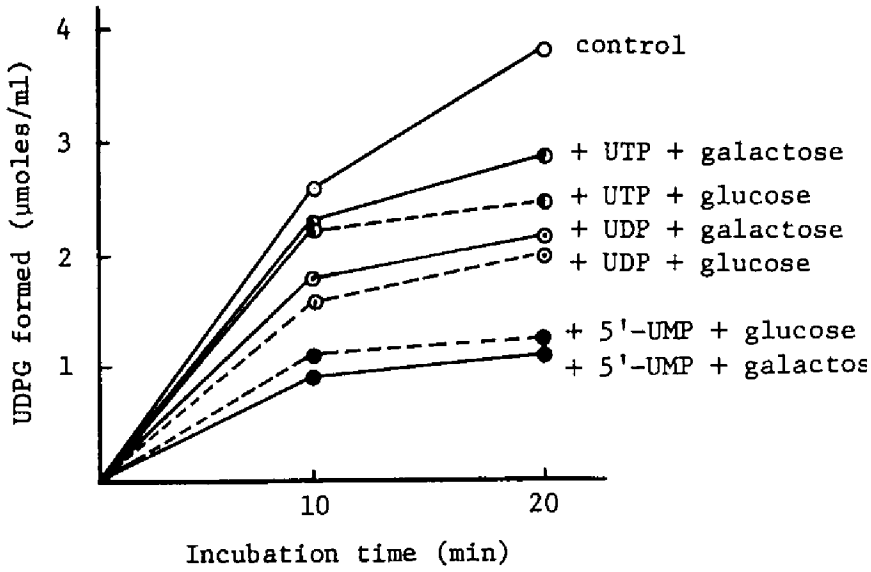


Fig. 7. Effect of Nucleotides and Sugars on UDPGal 4-Epimerase.

The control mixture (—o—) contained 9.7 μmoles of UDPGal, 5 μmoles of NAD, 200 μmoles of glycine buffer (pH 8.8) and 1.55 mg protein of enzyme in a final volume of 1 ml. To the control, nucleotides (each 10 μmoles/ml) and sugars (each 100 μmoles/ml) were added in combinations indicated in the figure. They were preincubated with enzyme, NAD and buffer at 30°C for 15 min, followed by addition of UDPGal.

DISCUSSION

Evidence obtained in a number of laboratories has shown that the UDPGal 4-epimerases obtained from various sources require NAD as a cofactor. The enzymes isolated from mammalian sources require the addition of a catalytic amount of NAD for the reaction,[26,55] whereas those from *S. fragilis* [30,50] and *E. coli*[53] do not require

NAD, since the coenzyme binds tightly to their enzyme protein. The present work showed that a partially purified epimerase from *Torulopsis candida* grown on a lactose medium was highly stimulated by the addition of exogenous NAD for the full activity. The *Km* for NAD of the yeast enzyme (1.4×10^{-4} M) was higher than those of calf liver (2×10^{-7} M) and bovine mammary gland (5×10^{-7} M). It appears likely that the *Torulopsis* enzyme contains loosely-bound NAD to its apoenzyme, unlike those from *S. fragilis* and *E. coli*. However, further investigation should be performed to clarify whether the yeast enzyme does really contain NAD in its native protein. The observation that the activity of the yeast epimerase undergoes a strong inhibition by incubation with 5'-UMP and galactose would suggest that different structural forms of the enzyme may exist as was reported in the purified epimerase of *S. fragilis* in the presence of 5'-UMP and certain specific sugars.[47] Kalckar *et al.*[48] also reported that native enzyme preparations from *S. fragilis* were largely composed of NAD-epimerase with only 15% to 20% in the form of fluorescent NADH-epimerase and that the addition of 5'-UMP and galactose was found to transform the epimerase into the highly fluorescent reduced form (NADH) of epimerase which was in an inactive state. They assumed that this inactivation of the reduced enzyme might be due to the reduced state of the prosthetic group brought about by the substrate analogues such as 5'-UMP and galctose, or

might be due to an alteration of the protein conformation.[48]

It is assumed that the inactivation of the *Torulopsis* enzyme may possibly be brought about in a similar way as was observed in the *Saccharomyces* enzyme. The present investigation will also strongly support the consideration in the previous section that the efficient accumulation of UDPGal from 5'-UMP and galactose by dried cells of *T. candida* may result from the specific inhibition of UDPGal 4-epimerase activity by the fermentation substrates, 5'-UMP and galactose.

SUMMARY

A partially purified preparation of UDPGal 4-epimerase from lactose-grown *Torulopsis candida* was found to require exogenous NAD for the full activity. The *Km* for NAD was 1.4×10^{-4} M, showing a relatively low affinity as compared to the enzymes from mammalian sources. The enzyme activity was remarkably inhibited by incubation with 5'-UMP, provided that galactose was also present. The concentration of 5'-UMP seemed more critical than that of galactose on the inhibition; it occurred in the presence of low concentration of 5'-UMP, provided that galactose was present enough. The catalytic activity was almost recovered by a short dialysis of the enzyme preparations preincubated with 5'-UMP and galactose. A strong inactivation of the enzyme activity was also found by the combination of 5'-UMP and glucose.

Chapter II. Fermentative Production of UDP-N-Acetyl-glucosamine by Yeasts

INTRODUCTION

UDPAG is known to participate in the biosynthesis of polymeric substances such as bacterial cell wall peptidoglycan and lipopolysaccharide, and chitin material of some fungi. This uridine coenzyme was first separated and identified from baker's yeast as a concomitant of UDPG preparations,[13] and has been isolated from a variety of organisms including bacteria,[60,61] fungi[62,63] plants[64] and animals.[65,66] In spite of its wide distribution and biological importance, UDPAG has been prepared at the present time by chemical synthesis[10] and by extraction from toluene-autolyzed cells of baker's yeast which contain small and variable amounts of the nucleotide.[13,15]

In the previous chapter, the author has reported a method for the fermentative production of biochemically important uridine coenzyme, UDPGal, from 5'-UMP and galactose with dried cells of *Torulopsis candida* IFO 0768 in the presence of high concentration of inorganic phosphate.

The present study[67-69] deals with a new preparative method for UDPAG from 5'-UMP and glucosamine by the fermentative process of hexose by dried cells of baker's yeast. The isolation and identification of UDPAG were described, and various factors influencing

UDPAG fermentation were also examined.

MATERIALS AND METHODS

Materials. 5'-UMP sodium salt was kindly supplied by Takeda Chemical Industries, Ltd., and Tanabe Seiyaku Co., Ltd. UDPG, UDP and UTP were prepared by the method reported previously.[22] UDPAG was purchased from Boehringer and Soehne GmbH, Mannheim. Prostatic phosphomonoesterase was generously given by Dr. Y. Sugino of Institute for Virus Research, Kyoto University. Bull seminal 5'-nucleotidase was kindly given by the Research Laboratories of Takeda Chemical Industries, Ltd.

Microorganisms and cultivation. Baker's yeast obtained from Oriental Yeast Co., Ltd., was air-dried at room temperature for 24-36 hr on a filter paper, followed by drying overnight under reduced pressure in a desiccator containing NaOH. For the preparation of well-dried cells, they were dried again over P_2O_5 overnight under reduced pressure. Other strains of yeasts were cultivated in a medium consisted of 5% glucose, 0.5% peptone, 0.2% yeast extract, 0.2% KH_2PO_4, 0.2% $(NH_4)_2HPO_4$, and 0.1% $MgSO_4 \cdot 7H_2O$. They were grown at 28°C for 24-48 hr on a reciprocal shaker with 2 liter-shaking flask containing 500 ml of the medium. The inoculum size was 1% of 24 hr culture grown on the same medium. The cells harvested by centrifugation were washed twice with tap water, dried at room tempera-

ture for 24 hr, and desiccated overnight under reduced pressure. The dried cells were kept at -20°C until used.

Staphylococcus aureus IFO 3060 was used for the preparation and purification of UDPAG pyrophosphorylase. The culture medium contained 1% meat extract, 1% peptone, 1% glucose and 0.5% NaCl (pH 7.0). The cultivation was carried out at 28°C for 18 hr aerobically with 2 liter-shaking flask containing 500 ml of the above medium. Staphylococcal UDPAG pyrophosphorylase was partially purified by the method of Strominger and Smith.[70]

Analyses. UDPAG, UDPG, UTP, UDP, 5'-UMP and uridine were determined by paper chromatography as described in the first section of the previous chapter. Nucleotides were separated by column chromatography using Dowex 1 x 2 (Cl^- form) according to the method of Cohn and Carter.[32] Paper chromatography of hexosamines was carried out with ethyl acetate-pyridine-H_2O (4:2:4, v/v) and ethyl acetate-pyridine-NH_4OH-H_2O (10:5:3:3, v/v),[33] and the Morgan-Elson reagent was sprayed for sugar detection.[71] For the identification and determination of N-acetylglucosamine, colorimetric method was performed according to Reissig *et al.*[72] Total phosphate was assayed by determining the inorganic phosphate after digestion of the sample with sulfuric acid by the method of Fiske-Subbarow.[73]

RESULTS

Reaction system for UDPAG formation

In order to investigate whether UDPAG is formed from 5'-UMP by dried cells of baker's yeast, various reaction systems were devised as shown in Table I. It was found that the phosphorylation of added 5'-UMP to UDP and UTP took place in all of the reaction systems. The time course study showed that UDPG was formed with incubation time in fructose-containing systems (tube No. 2,3,4 and 5). However, in the reaction systems of tube No. 4 and 5, in which both fructose and glucosamine were present, another ultraviolet absorbing spot different from UDPG was apparently detected on a paper chromatogram. The *Rf* value of the spot was nearly identical with that of authentic UDPAG.

TABLE I. REACTION SYSTEM FOR UDPAG FORMATION BY DRIED CELLS OF BAKER'S YEAST

Additions (μmoles)		Tube No.				
		(1)	(2)	(3)	(4)	(5)
5'-UMP Na salt	50	+	+	+	+	+
Fructose	500	-	+	+	+	+
Glutamine	100	-	-	+	+	-
Glucosamine	100	-	-	-	+	+

Each tube contained 10 μmoles of $MgCl_2$, 500 μmoles of potassium phosphate buffer (pH 7.4) and 300 mg of dried cells of baker's yeast with additions indicated in the table in a total volume of 3 ml. The reaction was carried out at 28°C for several hours by shaking (280 rpm). +, added; -, not added.

A typical time course of the reaction in tube No. 5 is shown in Fig. 1. At an early stage of the reaction, a rapid decrease of 5'-UMP occurred, followed by an accumulation of large amount of UDP and UTP. Following the accumulation of nuclecside polyphosphate, UDPAG compound began to increase gradually and reached a maximum after 10 hr incubation in a yield of about 40% based on 5'-UMP added. Further incubation, however, brought about a decrease of the nucleotide and a simultaneous appearance of 5'-UMP.

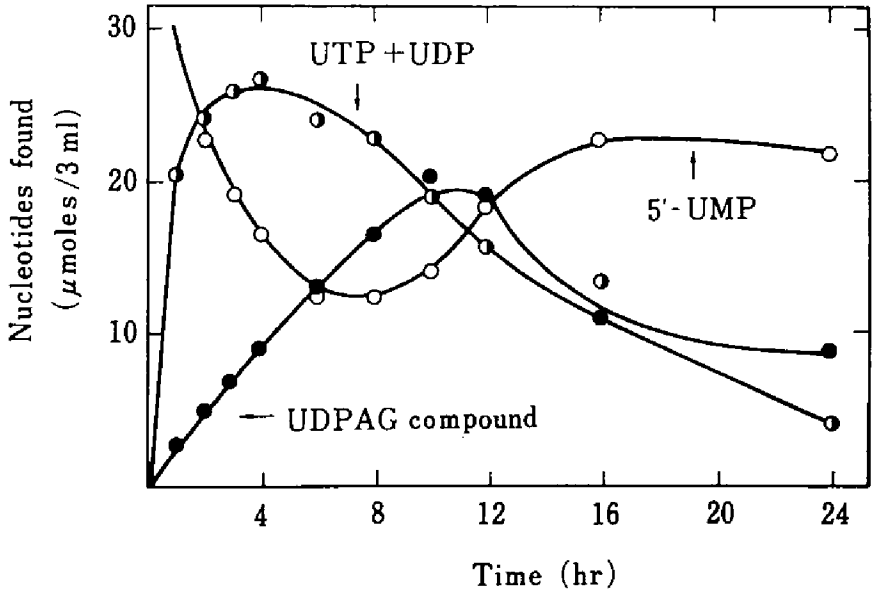


Fig. 1. Conversion of 5'-UMP to UDPAG and UTP + UDP by Dried Cells of Baker's Yeast.

The reaction system was tube No. 5 described in Table I. At the indicated intervals, nucleotides were determined by paper chromatography with a solvent of 95% ethanol-M ammonium acetate (7.5:3, pH 7.5).

It is possible that the UDPAG compound formed under the above condition may be similar to a UDPX compound of Paladini and Leloir[12] which was contaminated in their UDPG preparations from yeast and identified later as UDPAG.[13]

Isolation and identification of UDPAG

For the isolation and identification of UDPAG, a large scale incubation was performed. The reaction mixture contained 20 mmoles of fructose, 20 mmoles of glucosamine hydrochloride, 2 mmoles of 5'-UMP, 20 mmoles of potassium phosphate buffer (pH 7.4), 0.5 mmole of $MgCl_2$ and 10 g of dried cells of baker's yeast in a total volume of 100 ml. The reaction was carried out at 28°C for 10 hr in a 500 ml-shaking flask by continuous shaking. The reaction was terminated by immersing the flask into boiling water for 5 min, and cooled. The supernatant solution was treated with charcoal, followed by elution with 50% ethanol solution containing 5% NH_4OH. The eluate was concentrated by evaporation under reduced pressure and applied to a column of Dowex 1 x 2 (Cl^- form). The elution was carried out with 0.01 N HCl containing increasing amounts of NaCl.

Figure 2 shows the elution pattern of the nucleotides. It was found that 6 fractions of nucleotides were separated, each of which was identified by paper chromatography and UV ratio as follows: A, unidentified; B, 5'-UMP; C, UDPAG compound; D, UDPG; E, UDP; F, UTP. When an aliquot of each fraction was hydrolyzed at 100°C for

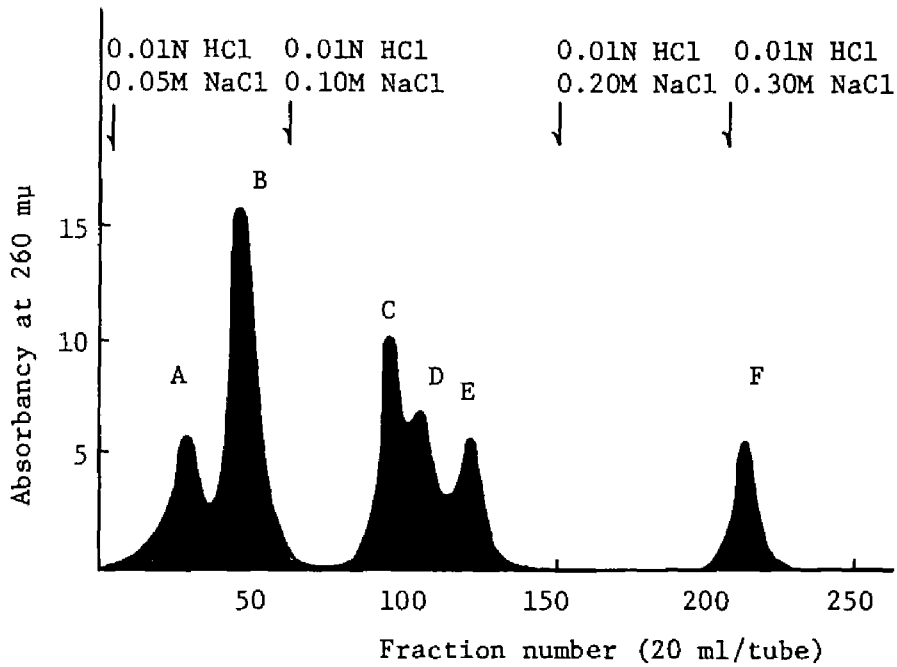


Fig. 2. Separation of Nucleotides by Column Chromatography with Dowex 1 x 2 (Cl^- Form).

The concentrated solution containing about 1260 μmoles of nucleotides as uridine was applied to the column (2.5 x 22 cm). The column was washed with distilled water until the absorbancy of the eluate at 260 mμ fell below 0.1. The elution was done with HCl-NaCl mixture at the flow rate of 4 ml/min.

15 min in 0.01 N HCl and was subjected to color reaction specific for acetylhexosamine according to Reissig *et al.*,[72] only the fraction (C) showed a positive color reaction. Then, the fraction was collected and adsorbed on charcoal, followed by elution with ammoniacal ethanol as described above. The eluate was concentrated by evaporation at 30°C under reduced pressure and lyophilized. In some cases

the nucleotide was precipitated as calcium salt by the addition of a saturated solution of calcium chloride in ethanol, followed by several volumes of ethanol until no more precipitation occurred.

The isolated nucleotide was found to have E_{max} at 262 mμ and E_{min} at 230 mμ in 0.01 N HCl, the absorption spectrum being identical with that of uridine. By paper chromatography of the nucleotide with two different solvent systems, a single spot was obtained with the same $R_{uridine}$ value as authentic UDPAG, and UDP and UMP were formed from the nucleotide by acid-hydrolysis. The result is shown in Table II.

TABLE II. $R_{uridine}$ VALUES OF ISOLATED NUCLEOTIDE AND ITS ACID-HYDROLYZATES

Compounds	$R_{uridine}$ values	
	Solvent I	Solvent II
Authentic UDPAG	0.58	0.40
" UDPG	0.44	0.27
" 5'-UMP	0.33	0.57
" UDP	0.18	-
" UTP	0.11	0.11
Sample A	0.58	0.39
" B	0.18	-
" C	0.31	0.56

Sample A; isolated nucleotide, B; acid-hydrolyzate (0.01N HCl, 100°C, 10 min), C; acid-hydrolyzate (1N HCl, 100°C, 20 min)
Solvent I; 95% ethanol-M ammonium acetate (7.5:3, pH 7.5)
Solvent II; 95% ethanol-M ammonium acetate buffer (7.5:3, pH 3.8)

The uridine monophosphate liberated from the isolated nucleotide was hydrolyzed by bull seminal 5'-nucleotidase,[74] yielding equal amounts of inorganic phosphorus and a compound having the same *Rf* value as uridine. For the determination of the sugar moiety of the isolated nucleotide, it was hydrolyzed in 0.01 N HCl at 100°C for 10 min and the hydrolyzate was submitted to paper chromatography with ethyl acetate-pyridine-NH_4OH-H_2O (10:5:3:3, v/v) and Morgan-Elson spray reagent. A single purple-red spot was detected with the same *Rf* value as authentic N-acetylglucosamine, whereas a cherry-red spot with the same *Rf* value as authentic glucosamine was detected when hydrolyzed in 2 N HCl at 100°C for 2 hr. The colored product of the sugar moiety liberated on mild hydrolysis, when treated with alkaline Ehrlich's reagent, had maximal absorption at 545 mμ and 585 mμ, and minimal absorption at 570 mμ, the spectrum being specific for authentic N-acetylglucosamine.[72]

It was also found that one mole of the isolated nucleotide calculated as uridine had two moles of phosphorus, one of which was acid-labile, and one mole of N-acetylglucosamine. The results were well identical with theoretical values of authentic UDPAG.

The biological activity of the isolated nucleotide was investigated with UDPAG pyrophosphorylase purified from *Staphylococcus aureus*.[70] Table III shows that the isolated nucleotide is enzymatically degraded to UTP only in the presence of pyrophosphate, but

not in the absence of pyrophosphate. The amount of N-acetylglucosamine formed after treatment with prostatic phosphomonoesterase was nearly equal to that of UTP. The results obtained here strongly suggest that UTP and N-acetylglucosamine-1-phosphate may be formed from the isolated nucleotide by pyrophosphorolysis with the bacterial enzyme.

TABLE III. PYROPHOSPHOROLYTIC CLEAVAGE OF ISOLATED UDPAG BY STAPHYLOCOCCAL UDPAG PYROPHOSPHORYLASE

Reaction system	UDPAG consumed (μmoles)	UTP formed (μmoles)	N-Acetylglucosamine formed (μmoles)
Isolated UDPAG	trace	0	0
Isolated UDPAG + PPi	8.1	8.7	7.6

The incubation mixture contained 13.4 μmoles of the isolated UDPAG, 100 μmoles of sodium pyrophosphate, 100 μmoles of Tris buffer (pH 7.2), 5 μmoles of $MgCl_2$ and 2.1 mg protein of staphylococcal UDPAG pyrophosphorylase in a total volume of 5 ml. As a control, pyrophosphate was omitted from the incubation mixture. After incubation at 37°C for 60 min, the reaction mixture was chromatographed with neutral ethanol-ammonium acetate solvent to determine UDPAG and UTP. N-Acetylglucosamine was determined after incubation with prostatic phosphatase. The incubation mixture with the phosphatase contained 0.55 ml of the above reaction solution, 100 μmoles of acetate buffer (pH 5.0), 1 μmole of $MgCl_2$ and 0.05 ml of prostatic phosphatase in a total volume of 1.2 ml. The incubation was carried out at 37°C for 60 min.

The IR spectra of the isolated nucleotide and authentic UDPAG were compared and found to be identical as shown in Fig. 3.

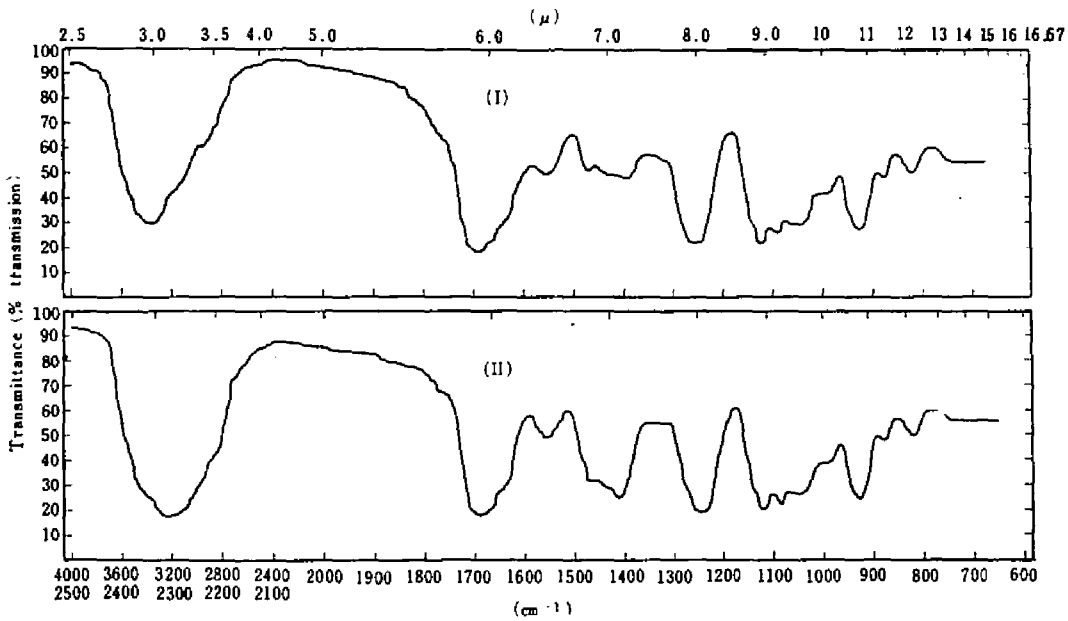


Fig. 3. IR Spectra of Isolated Nucleotide and Authentic UDPAG.

I: Authentic UDPAG, II: Isolated nucleotide

The NMR spectrum of the isolated nucleotide is shown in Fig. 4. The signals show the presence of protons of H-5 and H-6 in uracil, H'-1 in ribose, H''-1 in glucosamine and methyl protons in N-acetyl residue. The result was in good agreement with that reported by Onodera and Hirano.[75] *Anal.* Found C, 27.63; H, 4.52; N, 5.58%. Calcd. for $C_{17}H_{25}N_3O_{17}P_2Ca \cdot 5H_2O$: C, 27.75; H, 4.76; N, 5.71%.

From the results described above, it was concluded that the

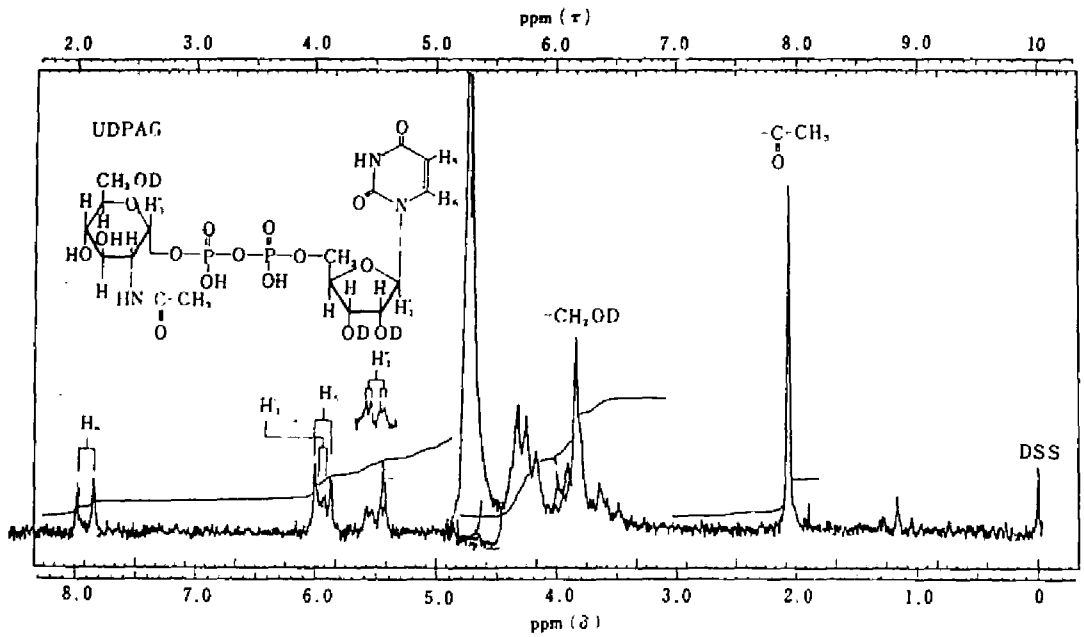


Fig. 4. NMR Spectrum of Isolated Nucleotide.

The spectrum was recorded on a Varian A-60 spectrometer at 60 MHz in deutrium oxide with DSS as internal standard.

isolated nucleotide was UDPAG.

Various factors influencing UDPAG fermentation

The standard reaction system for UDPAG formation is shown in Table IV. The effects of concentration of the reaction components, pH, sugar specificity and the water content of air-dried cells on the accumulation of UDPAG were investigated.

Glucosamine concentration. Figure 5 showed that no UDPAG accu-

TABLE IV. STANDARD REACTION SYSTEM FOR UDPAG FORMATION BY BAKER'S YEAST

5'-UMP sodium salt	50 μmoles
Glucosamine hydrochloride	50 μmoles
Fructose	200 μmoles
Potassium phosphate buffer (pH 7.4)	500 μmoles
$MgCl_2 \cdot 6H_2O$	10 μmoles
Dried cells of baker's yeast	300 mg
Total volume except dried cells	3 ml

The reaction was started by the addition of dried cells. The shaking reaction (280 rpm) was carried out at 28°C for 8 and 12 hr in test tubes. The reaction was terminated by immersing the tubes in boiling water for 2 min and cooled immediately. The reaction mixture was centrifuged and an aliquot of the supernatant solution was submitted to paper chromatography with 95% ethanol-M ammonium acetate (7.5:3, pH 7.5).

mulated without the addition of glucosamine and that a maximal formation of UDPAG was attained at 40-50 μmoles of glucosamine/3 ml with a yield of 36% based on the initial 5'-UMP added. It seems, however, that the higher concentration of glucosamine tends to lower the formation of UDPAG.

Fructose concentration. A considerable amount of UDPAG (12 μmoles/3 ml) was formed even in the absence of fructose, but it increased by adding fructose exogenously. The final concentration of

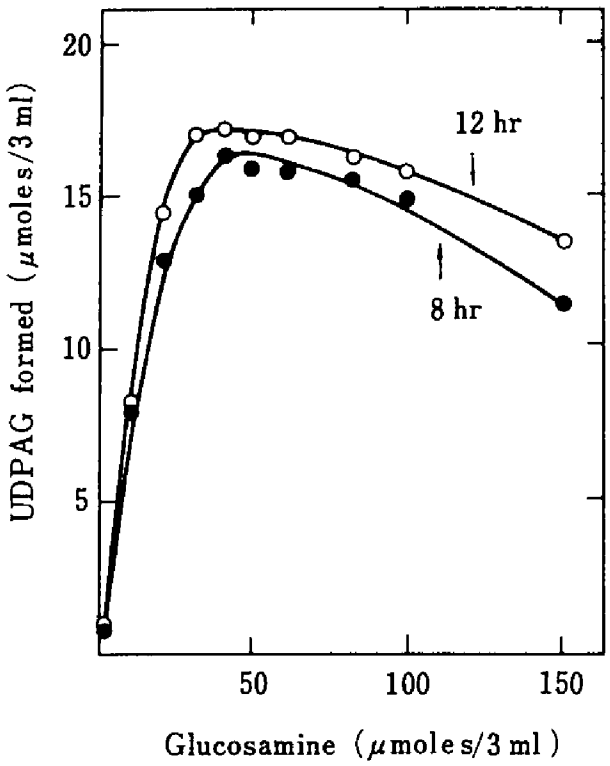


Fig. 5. Effect of Glucosamine Concentration on UDPAG Formation.

The reaction system was the same as described in Table IV except that glucosamine concentration was changed as shown in the figure.

fructose for a miximal formation was about 0.1 M (Fig. 6). The accumulation of UDPAG by the yeast in the absence of the hexose may possibly occur by the utilization of endogenous substances such as glycogen as energy sources.

5'-UMP concentration. The amount of UDPAG was found to increase with increasing amount of 5'-UMP up to 60 μmoles/3 ml of the reaction

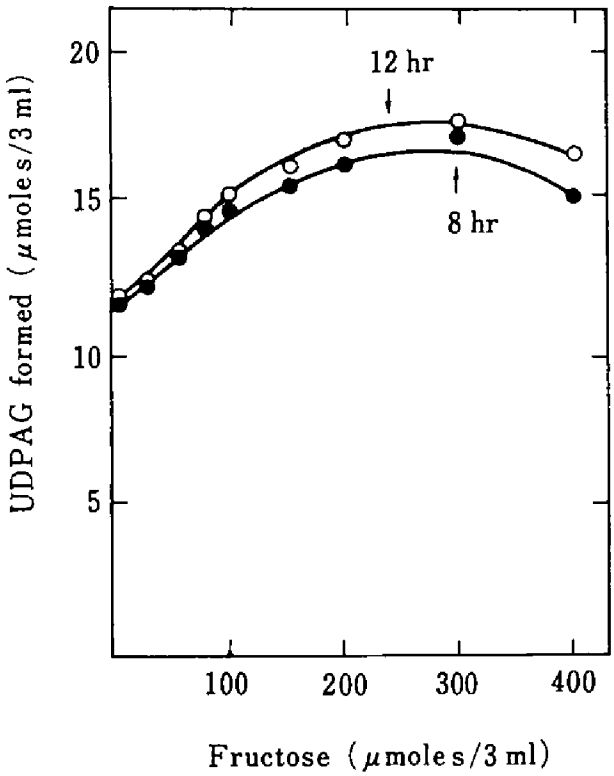


Fig. 6. Effect of Fructose Concentration on UDPAG Formation.

The reaction system was the same as described in Table IV except that fructose concentration was changed as shown in the figure.

mixture, but the yield based on the added nucleotide was rather lowered (Fig. 7).

Phosphate buffer concentration. The formation of UDPAG was remarkably influenced by the concentration of phosphate buffer used as shown in Fig. 8. A maximal accumulation was observed at the concentration of about 0.2 M. However, little amount of UDPAG was formed when phosphate buffer concentration was extremely high or low.

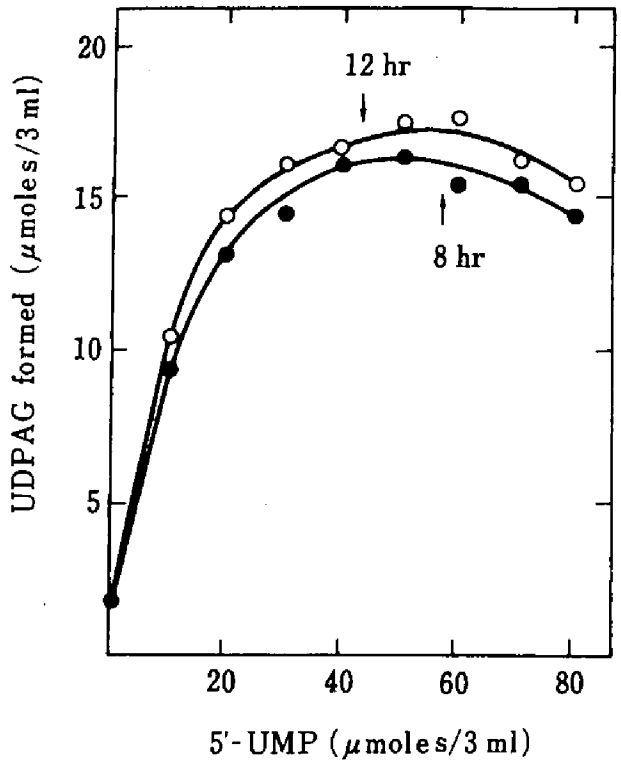


Fig. 7. Effect of 5'-UMP Concentration on UDPAG Formation.

The reaction system was the same as described in Table IV except that 5'-UMP concentration was changed as shown in the figure.

In the case of higher phosphate buffer concentration, the added nucleotide was not phosphorylated to UTP and, in the case of the lower concentration, the nucleotide was degraded to form uridine.

Effect of pH. The effect of initial pH of the reaction mixture on UDPAG formation is shown in Fig. 9. Alkaline condition was favored for UDPAG formation, the optimum pH being 8.0-8.5.

Dried cell concentration. The concentration of dried cells of

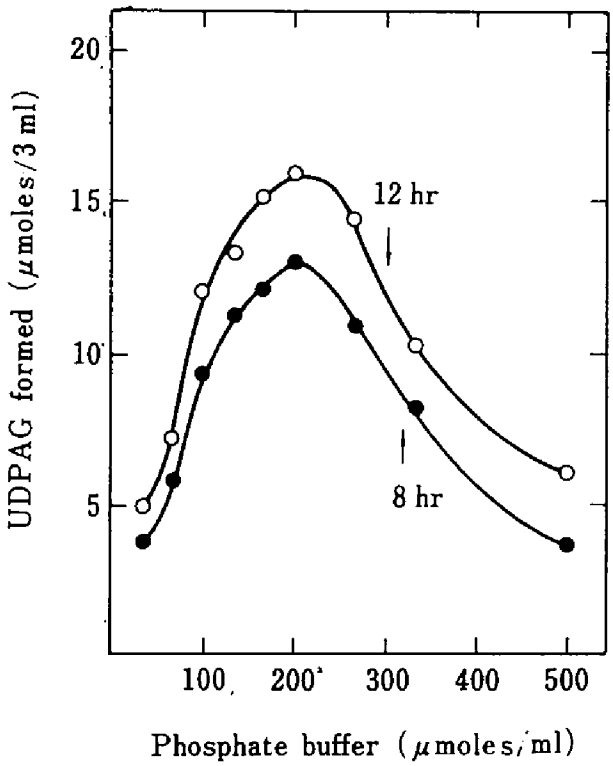


Fig. 8. Effect of Phosphate Buffer Concentration on UDPAG Formation.

The reaction system was the same as described in Table IV except that phosphate buffer concentration was changed as shown in the figure.

baker's yeast used as enzyme source was changed as shown in Fig. 10. UDPAG was increased linearly with amounts of dried cells up to about 200 mg/3 ml of the reaction mixture, but was decreased gradually as the concentration of dried cells became higher than 200 mg/3 ml. At the concentration of 600 mg/3 ml, the yield of UDPAG was 40%.

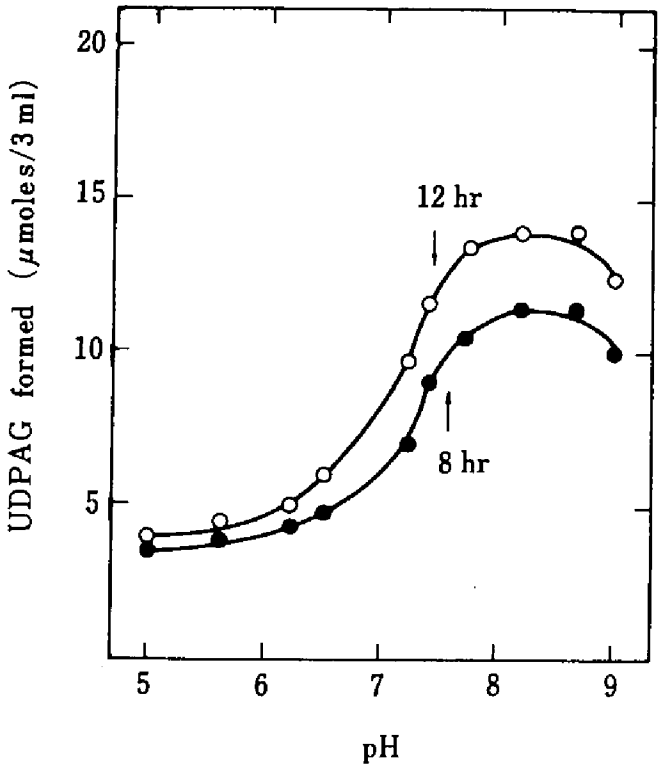


Fig. 9. Effect of pH on UDPAG Formation.

The reaction system was the same as described in Table IV except that pH of phosphate buffer was changed as shown in the figure. The pH (5.0, 5.6, 8.5 and 9.0) of phosphate buffer was adjusted with HCl or NaOH just before the experiment.

Effect of various carbohydrates. The effect of several mono- and disaccharides as energy sources on UDPAG formation was investigated (Table V). Although a considerable amount of UDPAG was found to be accumulated even when no carbohydrate was added, the amounts were inreased by the addition of fructose, glucose, mannose, maltose

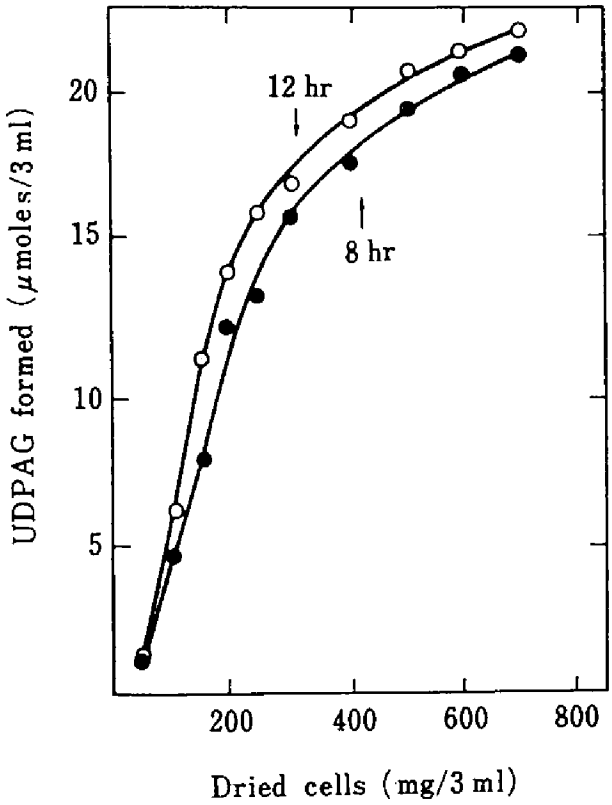


Fig. 10. Effect of Dried Cell Concentration on UDPAG Formation. The reaction system was the same as described in Table IV except that dried cell concentration was changed as shown in the figure.

and sucrose, respectively. It seemed that fructose and mannose were more effective than glucose and that maltose was the most suitable carbohydrate among the sugars tested.

Effect of hexosamines. UDPAG was formed only when glucosamine was used in the reaction mixture, though trace amounts of UDPAG were detected even in the absence of the hexosamine. Neither galactosa-

TABLE V. EFFECT OF VARIOUS CARBOHYDRATES ON UDPAG FORMATION

Carbohydrates incubated	UDPAG found (μmoles/3 ml)	
	8 hr	12 hr
None	7.6	10.3
D-Fructose*	9.6	14.7
D-Glucose	8.7	13.2
D-Glucose + D-Fructose	8.3	13.9
D-Mannose	9.7	14.9
D-Galactose	7.9	12.2
Maltose	10.1	16.4
Sucrose	9.6	13.6
Lactose	6.8	11.0

The reaction system was the same as described in Table IV except that 200 μmoles of various carbohydrates/3 ml of the reaction mixture were incubated in place of D-fructose.
* Standard reaction system.

mine nor N-acetylglucosamine could serve as a donor of the hoxosamine residue of UDPAG under the reaction condition (Table VI).

Effect of water content of dried cells. It has already been described in the previous chapter that the fermentative production of UDPGal by *Torulopsis candida* grown on a lactose medium was remarkably influenced by the water content of dried cells of the yeast used as enzyme source. The same phenomenon has recently been observed in the formation of GDPM by dried cells of baker's yeast.[24)]

As is shown in Table VII, the amount of UDPAG was obviously

TABLE VI. EFFECT OF HEXOSAMINES ON UDPAG FORMATION

Hexosamines incubated	UDPAG found (μmoles/3 ml)	
	8 hr	12 hr
None	0	trace
D-Glucosamine hydrochloride*	9.6	14.7
D-Galactosamine hydrochloride	0	trace
N-Acetyl-D-glucosamine	2.1	2.1

The reaction system was the same as described in Table IV except that 50 μmoles of D-galactosamine hydrochloride or N-acetyl-D-glucosamine/3 ml of the reaction mixture was incubated in place of D-glucosamine hydrochloride. * Standard reaction system.

increased with dried-cell preparations of baker's yeast which were dried again over P_2O_5. With the best preparation of 4.9% water content, the yield of UDPAG was about 66% after incubation of 12 hr. It seems necessary and favorable for the efficient formation of UDPAG to use well-dried cells of baker's yeast as in the case of the formation of UDPGal and GDPM.

Distribution of UDPAG forming activity in yeasts

The distribution of UDPAG forming activity was investigated with 42 strains of yeasts other than baker's yeast, the results being summarized in Table VIII. It is of interest that relatively strong activities are found to be distributed specifically among *Debaryo-*

TABLE VII. EFFECT OF WATER CONTENT OF CELL PREPARATIONS OF BAKER'S YEAST ON UDPAG FORMATION

Water content of dried cells (%)	Reaction pH	UDPAG found (μmoles/3 ml)		
		6 hr	8 hr	12 hr
(A) 12.0	7.2	8.3	9.1	13.3
	7.7	13.3	15.0	17.4
(B) 5.4	7.2	17.1	17.2	20.9
	7.7	16.0	18.5	21.5
(C) 4.9	7.2	20.6	22.7	33.0
	7.7	23.4	28.1	32.8

The reaction system was the same as described in Table IV except that 300 mg/3 ml of dried cells of different water content were incubated at pH 7.2 and 7.7 as shown above. The dried cells of lower water content ((B) and (C)) were prepared by drying (A) over P_2O_5 under reduced pressure.

myces species. In *D. subglobosus*, *D. globosus* and *D. cantarellii*, 33-40% of 5'-UMP was converted to UDPAG. Other strains of the same genus such as *D. japonicus* and *D. hansenii* accumulated UDPAG in about 14% yield. Small amounts of UDPAG were produced in *Saccharomyces lactis*, *Saccharomycodes ludwigii*, *Candida tropicalis* and *Torulopsis versatilis*. However, many other strains had little or no UDPAG forming activity; some of them degrading 5'-UMP to uridine, the other phosphorylating 5'-UMP to UDP and UTP under the reaction conditions.

TABLE VIII. FERMENTATIVE PRODUCTION OF UDPAG BY YEASTS

Strains	Reaction time (hr)	Products found (μmoles/ml)		
		UDPAG	Uridine	UTP + UDP
Endomyces hordei IFO 0104	4	0	6.3	0
	8	0	12.5	3.5
	12	0	15.3	2.0
Endomyces decipiens IFO 0102	4	0	11.8	2.8
	8	0	14.0	4.1
	12	0	17.5	3.1
Endomycopsis capsularis IFO 0672	4	0	16.0	2.9
	8	0	17.3	5.8
	12	0	17.1	trace
Zygosaccharomyces soja IFO 0495	4	0	2.4	0
	8	0	8.5	1.4
	12	0	15.3	0
Saccharomyces lactis IFO 1090	4	1.6	5.4	5.8
	8	2.5	8.1	3.0
	12	2.8	10.0	2.1
Saccharomyces fragilis IFO 0288	4	0	6.5	0
	8	0	13.3	0
	12	0	14.0	0
Saccharomyces carlsbergensis IFO 0641	4	0	2.8	0
	8	0	12.3	0
	12	0	7.8	2.6
Saccharomyces rouxii IAM 4369	4	0	0	0
	8	0	0	0
	12	0	8.5	0
Pichia polymorpha IFO 0195	4	0	1.9	0
	8	0	4.0	0
	12	0	3.8	0
Hansenula anomala AKU 4300	4	0	2.4	3.3
	8	0	4.0	0
	12	0	4.6	0
Hansenula capsulata IFO 0721	4	0	0	0
	8	0	4.5	0
	12	0	4.5	0

TABLE VIII. FERMENTATIVE PRODUCTION OF UDPAG BY YEASTS (continued)

Strains	Reaction time (hr)	Products found (μmoles/ml)		
		UDPAG	Uridine	UTP + UDP
Hansenula jadinii IFO 0987	4	0	0	0
	8	0	2.5	0
	12	0	4.1	0
Debaryomyces japonicus IFO 0039	4	trace	0	7.1
	8	3.9	4.5	4.5
	12	3.5	6.0	3.1
Debaryomyces hansenii IFO 0023	4	0	trace	0
	8	1.8	3.5	6.8
	12	3.1	1.3	9.6
Debaryomyces cantarellii IFO 1189	4	7.0	2.0	4.8
	8	8.3	3.1	4.1
	12	8.0	4.8	6.6
Debaryomyces castellii IFO 1359	4	0	5.3	8.1
	8	0	8.1	6.0
	12	0	10.0	trace
Debaryomyces globosus IFO 0016	4	5.1	5.4	4.5
	8	7.5	6.3	2.3
	12	9.0	6.8	0
Debaryomyces subglobosus IFO 0794	4	2.8	trace	8.1
	8	7.5	trace	4.0
	12	10.0	0	4.1
Saccharomycodes ludwigii AKU 4400	4	0	6.8	1.8
	8	1.6	12.0	2.1
	12	2.0	13.3	2.8
Hanseniaspora valbyensis IFO 0115	4	trace	8.3	2.4
	8	1.5	12.0	1.8
	12	2.0	17.5	1.1
Nadsonia elongata IFO 0665	4	0	4.9	2.5
	8	0	7.9	1.5
	12	0	12.8	3.4
Nadsonia fulvescens IFO 0666	4	0	19.6	trace
	8	0	17.2	trace
	12	0	16.0	trace

TABLE VIII. FERMENTATIVE PRODUCTION OF UDPAG BY YEASTS (continued)

Strains	Reaction time (hr)	Products found (μmoles/ml)		
		UDPAG	Uridine	UTP + UDP
Lipomyces lipoferus IFO 0673	4	trace	8.6	0
	8	trace	11.0	0
	12	trace	12.5	0
Sporobolomyces roseus IFO 1040	4	0	5.5	0
	8	0	7.6	0
	12	0	9.1	0
Sporobolomyces roseus IFO 1105	4	0	18.0	0
	8	0	17.8	0
	12	0	17.1	0
Cryptococcus albidus IFO 0378	4	0	8.3	1.8
	8	0	13.0	0
	12	trace	12.5	0
Cryptococcus laurentii IFO 0372	4	0	trace	trace
	8	0	3.5	4.3
	12	0	5.1	4.6
Torulopsis candida IFO 0768	4	1.4	trace	10.5
	8	-	1.6	11.5
	12	1.8	2.0	12.8
Torulopsis famata IFO 1084	4	0	8.4	0
	8	1.3	8.5	0
	12	0	13.8	0
Torulopsis versatilis IFO 0652	4	1.3	trace	8.5
	8	3.0	1.3	8.5
	12	2.3	5.8	3.1
Torulopsis sphaerica IFO 0648	4	0	5.5	trace
	8	0	9.3	trace
	12	trace	12.5	trace
Brettanomyces anomalus IFO 0642	4	trace	7.4	trace
	8	1.3	8.5	trace
	12	1.8	8.3	trace
Brettanomyces claussenii IFO 0627	4	0	5.8	trace
	8	0	7.3	trace
	12	0	8.4	trace

TABLE VIII. FERMENTATIVE PRODUCTION OF UDPAG BY YEASTS (continued)

Strains	Reaction time (hr)	Products found (μmoles/ml)		
		UDPAG	Uridine	UTP + UDP
Candida utilis IFO 0396	4	0	trace	trace
	8	0	6.5	trace
	12	0	trace	trace
Candida krusei IFO 0013	4	0	5.1	trace
	8	trace	6.5	2.1
	12	1.1	6.8	trace
Candida tropicalis IFO 0006	4	2.8	3.8	3.9
	8	1.9	4.4	trace
	12	trace	6.9	0
Candida humicola IFO 0760	4	0	7.1	0
	8	0	8.8	trace
	12	0	11.0	trace
Candida pseudotropicalis IFO 0617	4	0	8.6	0
	8	0	8.8	1.8
	12	1.1	12.8	trace
Candida intermedia IFO 0761	4	1.4	2.5	4.6
	8	trace	4.1	2.6
	12	trace	5.5	2.0
Kloeckera apiculata IFO 0154	4	0	12.5	0
	8	trace	16.5	0
	12	trace	16.3	0
Trigonopsis variabilis IFO 0671	4	0	8.1	0
	8	trace	12.8	0
	12	1.0	14.3	1.0
Rhodotorula glutinis IFO 0389	4	0	7.8	0
	8	0	11.3	0
	12	0	11.5	trace

The reaction system was the same as described in Table IV except that 75 μmoles of 5'-UMP, 600 μmoles of potassium phosphate buffer (pH 8.0) and 300 mg of dried cells of various yeasts were used per 3 ml of the reaction mixture.

DISCUSSION

The biological importance of UDPAG in the biosynthesis of bacterial cell walls has been elucidated since Park and Johnson[5] discovered UDP-N-acetylmuramic acid and UDP-N-acetylmuramic acid peptides in penicillin-treated *Staphylococcus aureus*. UDPAG has been prepared at the present time by chemical synthesis[10] and by extraction from toluene-autolyzed baker's yeast.[13,15]

In this report, a new preparative method of UDPAG is described. The uridine coenzyme was accumulated in the reaction system consisting of 5'-UMP, glucosamine, fructose, inorganic phosphate, magnesium ion and dried cells of baker's yeast as enzyme source. The final yield of UDPAG based on 5'-UMP added was about 40-66% when several fermentative conditions were optimized. The previous works[22-24,28] have shown that UDPG, UDPGal and GDPM are able to be prepared in high yields from corresponding nucleoside 5'-monophosphates by the fermentation and respiration of hexoses by various yeasts. The present method for the preparation of UDPAG also seems to be available for the practical purposes.

It is known that some yeasts including *Saccharomyces cerevisiae* contain chitin-like substances in their cell walls.[76] It seems to be an interesting problem to clarify the mechanism of UDPAG accumulation by baker's yeast under the specified conditions examined in this work, and to investigate the relation between the

content of yeast chitin and activity of UDPAG synthesis.

SUMMARY

The fermentative production of UDPAG from 5'-UMP and glucosamine by dried cells of baker's yeast was investigated. UDPAG was found to accumulate in a reaction system containing 5'-UMP, glucosamine, fructose, inorganic phosphate and magnesium ions with air-dried cells of baker's yeast as enzyme source. UDPAG was separated from the reaction mixture by means of anion exchange column chromatography and was identified by several biochemical methods.

The reaction conditions for the fermentative production of UDPAG were examined. The yield of UDPAG was about 40-66% based on 5'-UMP when fermentative conditions were optimized. The concentrations of glucosamine and potassium phosphate buffer, and pH as well as the water content of dried cells greatly affected the formation of UDPAG. The distribution of UDPAG forming activity among other yeasts was investigated. Relatively strong activities were found in some *Debaryomyces* species by which 33-40% of 5'-UMP was converted to UDPAG.

Chapter III. Fermentative Production of Cytidine Coenzymes by Yeasts

Section 1. Fermentative Production of CDP-Choline

INTRODUCTION

CDP-Choline was first synthesized chemically by Kennedy[77] and was found to be an important intermediate in the biosynthetic pathway of lipids.[78,79] This compound is widely distributed among animals, plants and microorganisms. Though some strains of yeasts contained large amounts of the cytidine coenzyme, it was only endogenously synthesized. By the application of the fermentative method of uridine coenzymes described in the previous chapters, the author has succeeded in converting 5'-CMP to CDP-choline in good yields.

This section[80,81] deals with the isolation and identification of CDP-choline formed under the fermentative condition of glucose by brewer's yeast. As the production of CDP-choline depended on many factors, the optimum conditions for the reaction were investigated. The author also describes the strains of yeasts which are able to convert 5'-CMP to CDP-choline in good yields.

MATERIALS AND METHODS

Materials. 5'-CMP sodium salt was kindly supplied by Takeda Chemical Industries, Ltd. Authentic CDP-choline was purchased

from Boehringer and Soehne GmbH, Mannheim. All other chemicals were commercial products. 5'-Nucleotidase was prepared from bull seminal plasma.[74] Lyophilized powder of snake venom (*Agkistrodon halys*, Mamushi) was used as nucleotide pyrophosphatase.[82] Prostatic non-specific phosphatase was kindly supplied by Dr. Y. Sugino of the Institute for Virus Research, Kyoto University.

Microorganisms and cultivation. Brewer's yeast (*Saccharomyces carlsbergensis*) was obtained from Kirin Brewery Co., Ltd. The wet yeast was dried by an electric fan for 12 hr at room temperature. The air-dried cells were completely dried over P_2O_5 for 24 hr *in vacuo*. The dried cells were kept at -20°C until used as enzyme source. Other strains of yeasts were obtained from the Institute for Fermentation, Osaka. Their growth medium contained 5.0% glucose, 0.5% peptone, 0.2% yeast extract, 0.2% $(NH_4)_2HPO_4$, 0.2% KH_2PO_4 and 0.1% $MgSO_4 \cdot 7H_2O$. Cultivation was carried out at 28°C for 24-48 hr on a reciprocal shaker with a 2 liter-shaking flask containing 500 ml of the medium. After harvesting by centrifugation, cells were washed three times with water, then dried with an electric fan to prepare air-dried cells.

Analyses. 5'-CMP, CDP, CTP and CDP-choline were determined by measuring their optical densities at 260 mμ after extracting their spots with 0.1 N HCl from paper chromatograms. Paper chromatography was carried out on Toyo Filter Paper No. 53 with 95%

ethanol-M ammonium acetate (7.5:3, pH 7.5).[3]

Reaction products were separated by column chromatography with Dowex 1 x 2 (Cl^- form) by the method of Cohn and Carter.[32] CDP-Choline was eluted with 0.001 N HCl, and 5'-CMP with 0.002 N HCl. Inorganic phosphate was determined by the method of Fiske and Subbarow[73] after the decomposition of samples by Nakamura's method,[83] or by other methods with various enzymes. CDP-Choline was identified by the results of various enzyme reactions, which were carried out at 37°C for 2 hr.

Reaction system. The composition of the reaction mixture is shown in Table I. The total reaction mixture of 2.0 ml was shaken at 28°C for several hours. The reaction was terminated by immersing the reaction tube in boiling water for 3 min. After addition of 2.0 ml of water, cell debris was removed by centrifugation at 3500 rpm for 5 min and the resulting supernatant solution was subjected to analysis. The screening mixture for CDP-choline formation by yeasts was the same as described in Table I except that 200 μmoles of potassium phosphate buffer (pH 8.0), 100 μmoles of phosphorylcholine and 100 mg of dried cells of various yeasts per ml of the reaction mixture were used.

RESULTS AND DISCUSSION

Conversion of 5'-CMP to CDP-choline by brewer's yeast

TABLE I. COMPOSITION OF REACTION MIXTURE

	per ml
5'-CMP sodium salt	20 μmoles
Glucose	400 μmoles
Phosphorylcholine calcium salt	50 μmoles
Potassium phosphate buffer (pH 7.0)	200 μmoles
$MgSO_4 \cdot 7H_2O$	12 μmoles
Air-dried cells of brewer's yeast	100 mg

Total reaction mixture of 2.0 ml was put in a test tube and reaction was carried out at 28°C with shaking.

After incubation with the reaction mixture shown in Table I, the products were analyzed by paper chromatography. Three UV-absorbing spots having *Rf* values different from that of 5'-CMP were detected on a paper chromatogram (Fig. 1). Two of them were identified, by their *Rf* values on the paper chromatogram, as CDP and CTP. The other showed the largest *Rf* value, which was identica with that of authentic CDP-choline. The third cytidine derivative was not synthesized when any one of 5'-CMP, phosphorylcholine, or the air-dried cells was omitted from the reaction mixture.

A typical time course of the reaction is shown in Fig. 2. In the earlier stages of the reaction, 5'-CMP is consumed rapidly in accordance with the formation of CDP and CTP. On longer incubation, CDP and CTP decrease with formation of the third cytidine derivative. The cytidine derivative accumulated was stable even

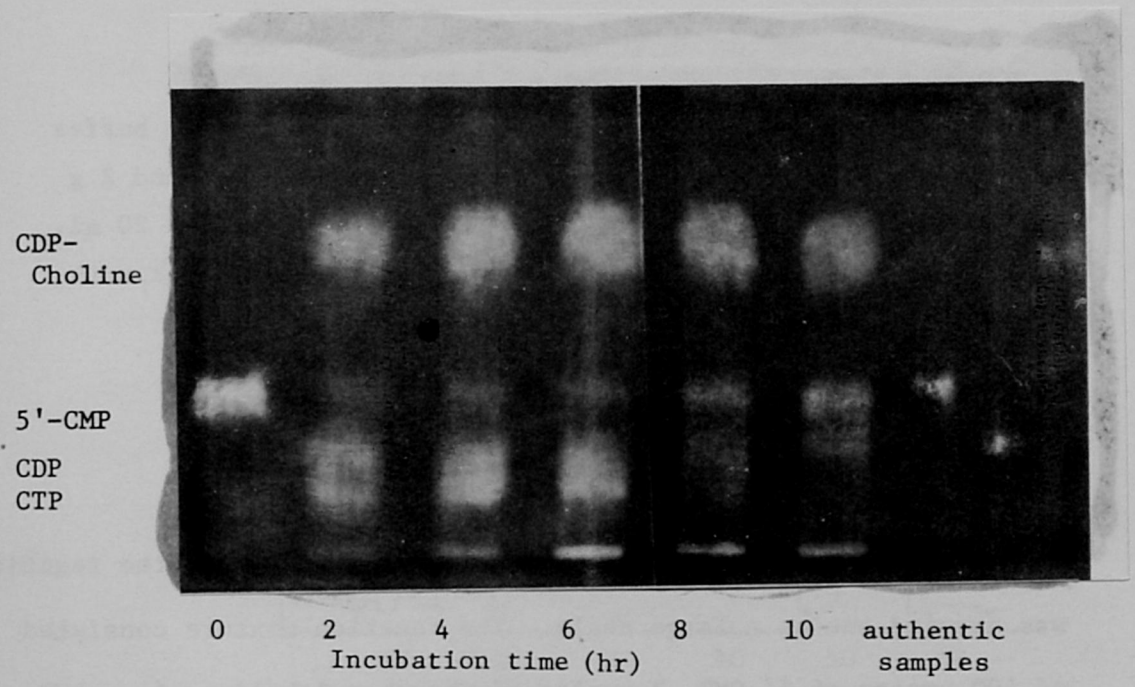


Fig. 1. Formation of CDP-Choline from 5'-CMP by Dried Cells of Brewer's Yeast.

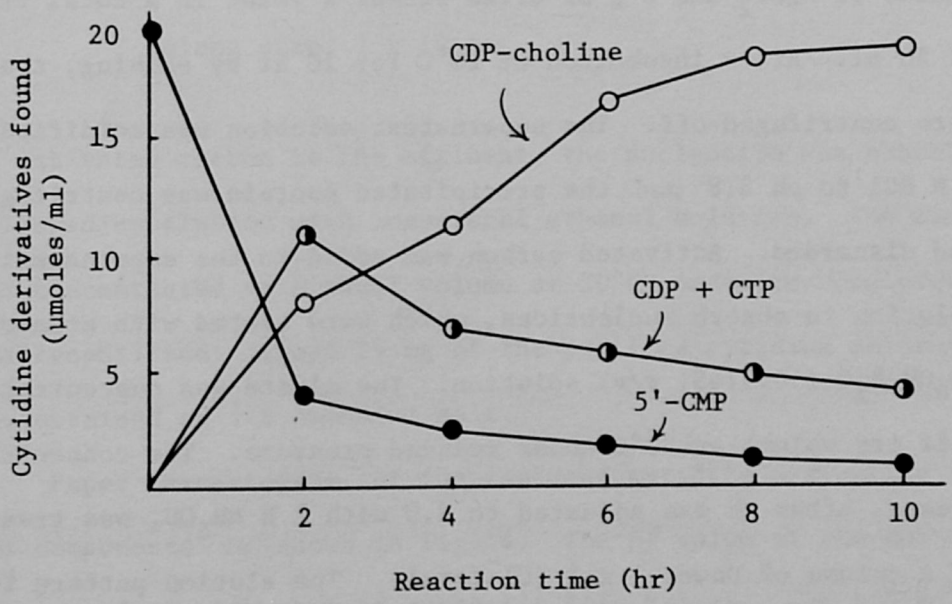


Fig. 2. Time Course of Formation of Cytidine Derivatives.

The reaction mixture consisted of 400 μmoles of 5'-CMP, 2 mmoles

of phosphorylcholine, 4 mmoles of potassium phosphate buffer (pH 7.0), 12 mmoles of glucose, 200 μmoles of $MgCl_2$ and 2 g of dried cells of brewer's yeast in a total volume of 20 ml. The reaction was carried out at 28°C in a 300 ml-flask with shaking.

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after 12 hr incubation.

Isolation and identification of CDP-choline

In order to isolate the third cytidine derivative, the reaction was carried out on a large scale. The reaction mixture consisted of 400 μmoles of 5'-CMP, 2 mmoles of phosphorylcholine, 4 mmoles of potassium phosphate buffer (pH 7.0), 12 mmoles of glucose, 200 μmoles of $MgCl_2$ and 2 g of dried brewer's yeast in a total volume of 20 ml. After incubation at 28°C for 10 hr by shaking, the cells were centrifuged off. The supernatant solution was acidified with 1 N HCl to pH 3.8 and the precipitated protein was centrifuged and discarded. Activated carbon was added to the supernatant solution to absorb nucleotides, which were eluted with ethanol-NH_4OH-H_2O (50:5:45, v/v) solution. The eluate was concentrated to half its volume at 30°C under reduced pressure. The concentrated eluate, after pH was adjusted to 8.0 with 1 N NH_4OH, was treated on a column of Dowex 1 x 2 (Cl^-form). The elution pattern for a typical small scale reaction mixture is shown in Fig. 3. The main cytidine derivative was eluted with 0.001 N HCl. By the addition

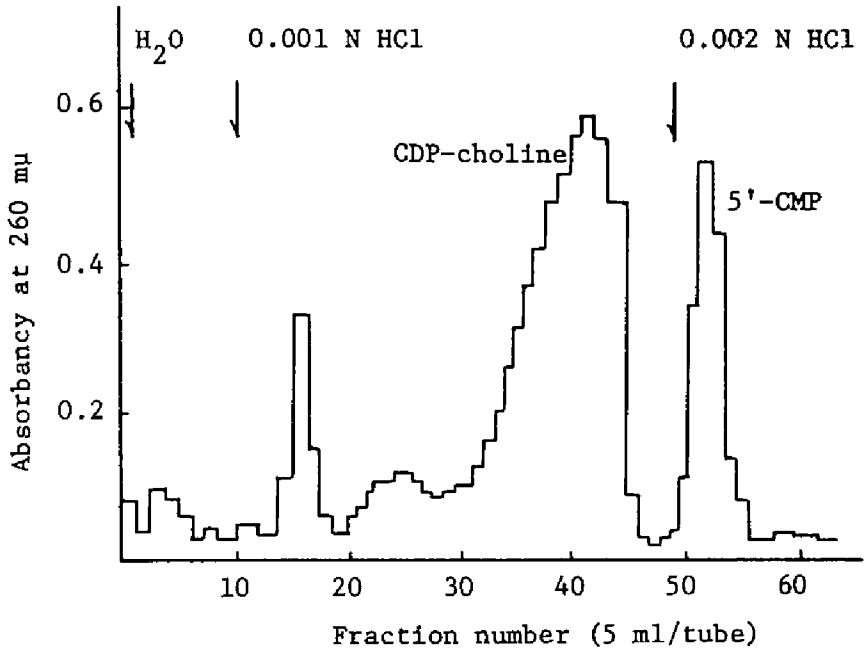

Fig. 3. Separation of CDP-Choline by Dowex 1 x 2 (Cl^- Form) Column Chromatography.

Column size: 1 x 19 cm

of activated carbon to the effluent, the nucleotide was absorbed, followed by elution with ammoniacal ethanol solution. The eluate was concentrated to a small volume at 30°C under reduced pressure and lyophilized. About 25 mg of the purified cytidine derivative was obtained as its ammonium salt.

Paper chromatogram of the isolated cytidine derivative and its components is shown in Fig. 4. The *Rf* value of the derivative was identical with that of authentic CDP-choline. The hydrolyzate (1 N HCl, 100°C, 80 min) of the sample did not contain free inorganic phosphate, but contained 5'-CMP and phosphorylcholine. The former

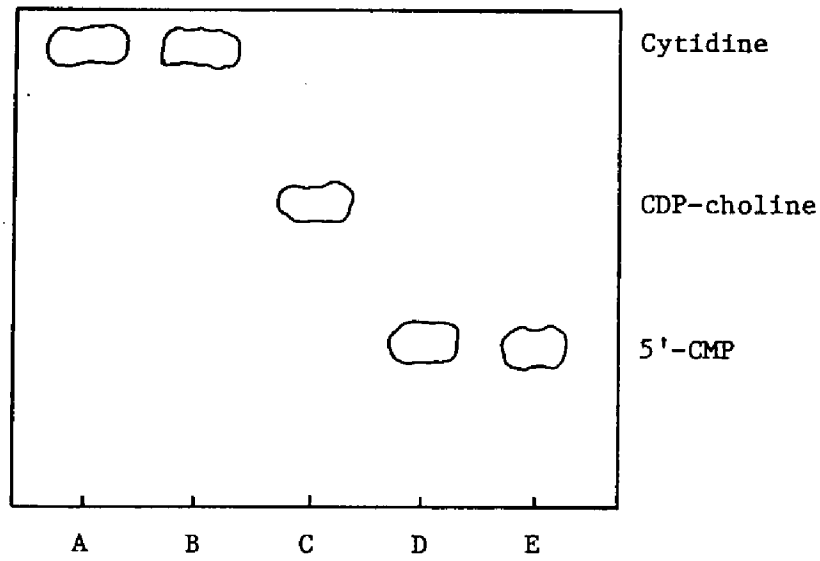

Fig. 4. Paper Chromatogram for Identification of Isolated CDP-Choline.

Solvent: 95% ethanol-M ammonium acetate (7.5:3, pH 7.5) A: authentic cytidine, B: acid-hydrolyzate of isolated CDP-choline treated with bull seminal 5'-nucleotidase, C: isolated CDP-choline, D: acid-hydrolyzate of isolated CDP-choline (1 N HCl, 100°C, 80 min), E: authentic 5'-CMP.

was confirmed by paper chromatography and by treatment with 5'-nucleotidase, and the latter was proved by spraying with Dragendorf reagent on paper chromatograms. On treatment with non-specific phosphatase the isolated phosphorylcholine released one mole of phosphorus per mole of choline, which was determined as reineckate by the method of Ackerman and Salmon.[84] Total phosphorus was determined after decomposition with sulfuric acid.[83] The deriva-

tive contained two moles of phosphorus per mole of cytidine. On treatment of the hydrolyzate with 5'-nucleotidase the derivative released one mole of phosphorus per mole of cytidine, whereas by treatment with non-specific phosphatase it released two moles of phsphorus. The derivative liberated one mole of phosphorus when treated with snake venom (*Agkistrodon halys*, Mamushi),[82] which is known to contain pyrophosphatase and 5'-nucleotidase. These experiments show that in this cytidine derivative 5'-CMP and phosphorylcholine are bound through pyrophosphate linkage. The results are summarized in Table II.

The identification of the derivative was further confirmed by physicochemical analyses. The IR and UV spectra of the derivative were identical with those of authentic CDP-choline. The NMR spectrum of the derivative is shown in Fig. 5. The signals show the presence of the protons in positions of 5 and 6 in the pyrimidine ring and of the protons of the methyl group attached to the quaternary ammonium in the choline. The ratio of the numbers of these protons was 1 : 1 : 9, which indicated the presence of the pyrimidine ring and choline in the ratio of 1 : 1. From these results, the cytidine derivative was identified as CDP-choline.

Factors affecting CDP-choline fermentation

As the fermentation of CDP-choline by brewer's yeast may be influenced by several factors, the optimum reaction conditions

TABLE II. IDENTIFICATION OF ISOLATED CDP-CHOLINE

(1) Total phosphates

	Pi found*
5'-CMP	1.02
CDP-choline	1.94
Sample	1.96

(2) Hydrolysis with HCl (1 N, 100°C 80 min)

	CMP found*
CDP-choline	1.01
Sample	0.93

(2-a) Treatment with 5'-nucleotidase (37°C, 2 hr)

	Pi found*
5'-CMP	0.93
CDP-choline	1.05
Hydrolyzate	1.08

(2-b) Treatment with non-specific phosphatase (37°C, 2 hr)

	Pi found
Phosphorylcholine	1.03**
CDP-choline	2.16*
Hydrolyzate	1.84*

(3) Treatment with snake venom (pyrophosphatase and 5'-nucleotidase)

	Pi found	Cytidine found*
Phosphorylcholine	0**	-
5'-CMP	1.28*	1.21
CDP-choline	0.91*	1.18
Sample	0.90*	0.87

(continued)

(4) Measurement of choline after separation of phosphorylcholine

	Pi found***
Phosphorylcholine	1.220
Sample	1.225

* μmoles/μmoles of sample calculated as cytidine from E_{260}

** μmoles/μmoles of substrate

*** μmoles/μmoles of choline

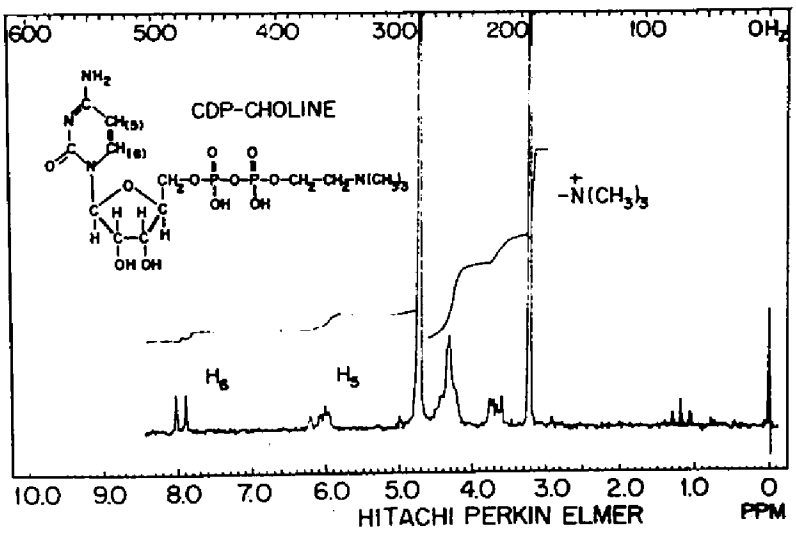

Fig. 5. NMR Spectrum of Isolated CDP-Choline.

were investigated with respect to pH and concentrations of substrates.

Effect of pH. The formation of CDP-choline proceeded efficiently at pH of more than 7.0, as shown in Fig. 6. Even at pH 9.74 the formation of CDP-choline was observed. These pH values were the initial ones, which decreased to 6.0-6.4 by the end of the reaction.

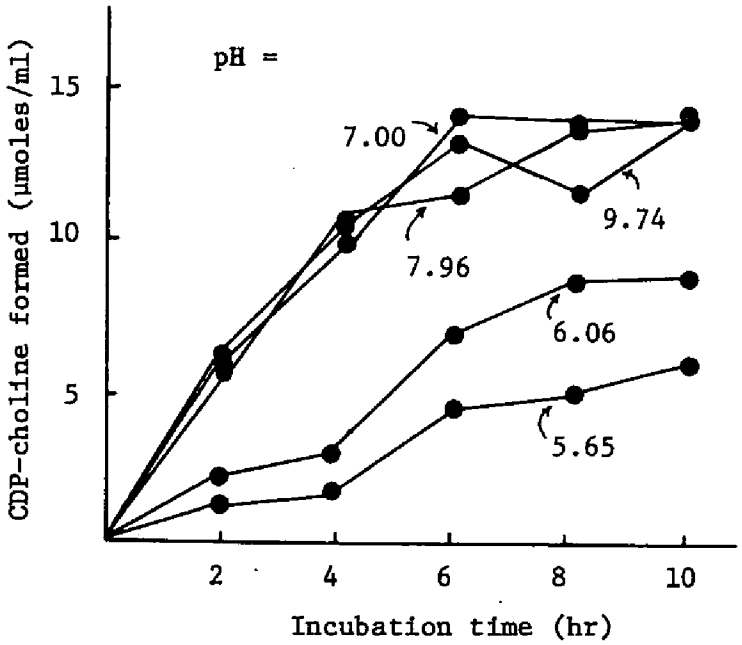

Fig. 6. Effect of pH on Formation of CDP-Choline.

The reaction system was the same as that described in Table I except that the pH of the phosphate buffer was varied as shown in the figure.

Glucose concentration. Glucose is one of the most important factors in this kind of energy-consuming reaction. The addition of 600-800 μmoles/ml of glucose brought about a high yield of CDP-choline (Fig. 7).

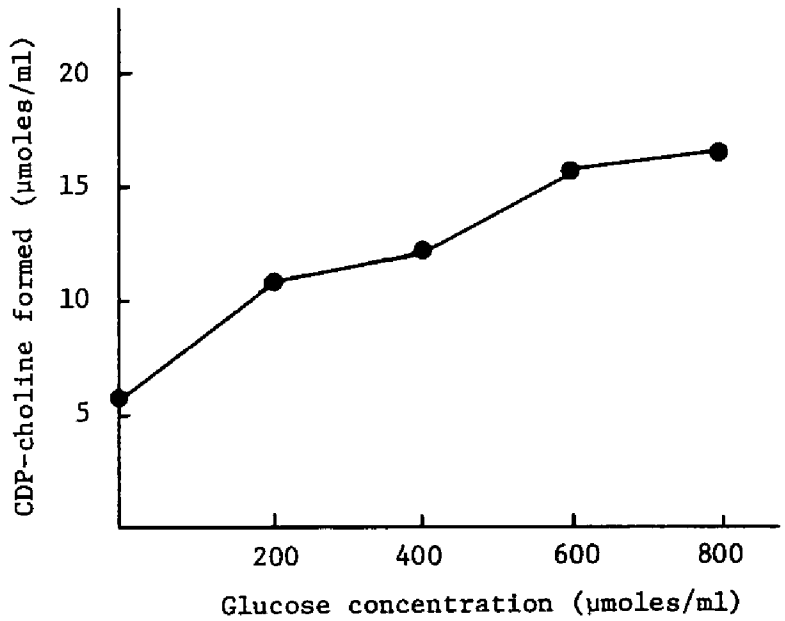

Fig. 7. Effect of Glucose Concentration on Formation of CDP-Choline.

The reaction system was the same as that described in Table I except that the glucose concentration was varied as shown in the figure. The incubation time was 7 hr.

Phosphate buffer concentration. In the case of the formation of uridine coenzymes described in the previous chapters, a relatively high concentration of phosphate buffer was the most decisive

element, since under ordinary (low) phosphate concentrations, nucleoside-5'-monophosphate was not phosphorylated, but rather decomposed. Figure 8 shows that the optimum phosphate buffer concentration for CDP-choline formation is near 200 μmoles/ml.

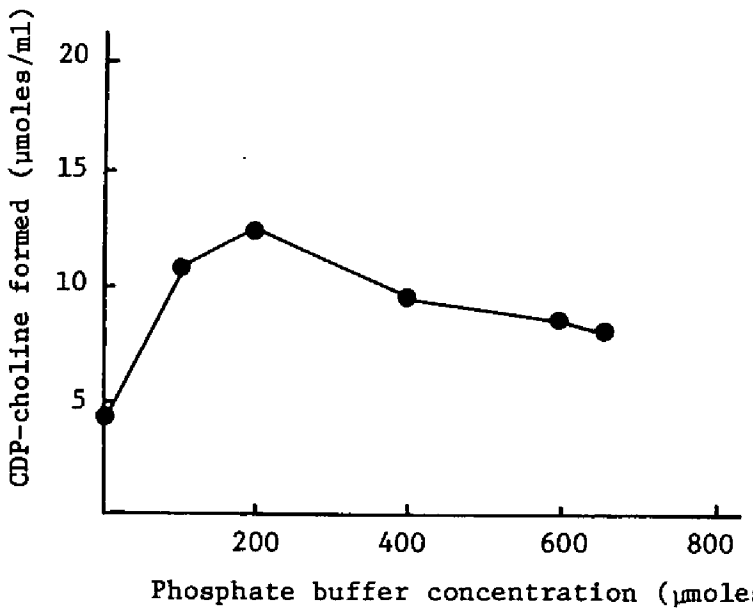

Fig. 8. Effect of Phosphate Buffer Concentration on Formation of CDP-Choline.

The reaction system was the same as that described in Table I except that the concentration of potassium phosphate buffer (pH 7.0) was varied as shown in the figure. The incubation time was 7 hr.

Phosphorylcholine concentration. A preliminary experiment showed that phosphorylcholine was the most effective substrate

for the fermentative production of CDP-choline. Then, the effect of phosphorylcholine concentration was investigated. As is shown in Fig. 9, the increase of phosphorylcholine concentration accelerated the formation of CDP-choline. The data show that 100 μmoles per ml of phosphorylcholine is still more effective for CDP-choline formation. Without phosphorylcholine, little formation of the cytidine coenzyme occurred.

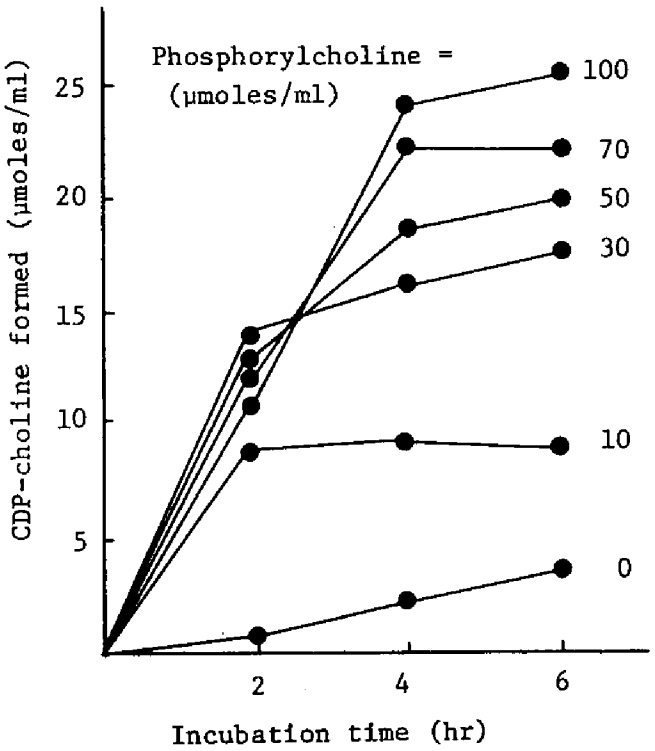

Fig. 9. Effect of Phosphorylcholine Concentration on Formation of CDP-Choline.

The reaction system was the same as that described in Table

I except that 30 μmoles/ml of 5'-CMP and the indicated amounts of phosphorylcholine were incubated.

Distribution of CDP-choline accumulating activity in yeasts

All the experiments described above were carried out with air-dried cells of brewer's yeast. Subsequently, the activity for formation of CDP-choline was investigated among various yeasts under the conditions described in Materials and Methods. Table I indicated that 33 strains (76.7%) out of 43 strains tested had the ability to form CDP-choline, and that in particular 8 strains (18.6%) converted more than 50% of available 5'-CMP to CDP-choline. *Saccharomyces rouxii* IAM 4309 and *Zygosaccharomyces soja* (*Saccharomyces rouxii*) IFO 0495 as well as brewer's yeast formed CDP-choline in good yields (about 80%) in the later stages of the reaction.

TABLE III. DISTRIBUTION OF CDP-CHOLINE FORMING ACTIVITY IN YEASTS

Strains	Reaction time (hr)	Products and substrate(μmoles/ml)		
		CDP-choline	5'-CMP	CDP + CTP
Saccharomyces fragilis IFO 0288	4	3.60	7.20	7.12
	8	4.68	9.60	7.88
	12	3.80	11.60	8.60
Saccharomyces carlsbergensis IFO 0641	4	10.20	2.60	6.56
	8	11.80	3.12	5.28
	12	11.40	3.72	6.88
Saccharomyces rouxii IAM 4309	4	14.20	2.68	6.88
	8	15.52	5.72	3.68
	12	16.40	8.20	3.20
Saccharomyces lactis IFO 1090	4	4.24	8.00	2.76
	8	8.40	1.36	5.72
	12	6.80	0.92	6.12
Endomyces hordei IFO 0104	4	1.80	2.48	3.28
	8	2.80	2.96	trace
	12	3.00	2.00	trace
Endomyces decipiens IFO 0102	4	0	11.10	0
	8	0	8.20	0
	12	0	10.80	0
Zygosaccharomyces soja IFO 0495	4	4.80	2.80	11.30
	8	12.60	4.00	6.24
	12	16.20	2.40	3.76
Pichia polymorpha IFO 0195	4	trace	16.80	trace
	8	trace	16.00	trace
	12	trace	16.80	trace
Endomycopsis capsularis IFO 0672	4	3.60	2.88	trace
	8	4.68	2.32	trace
	12	3.80	2.08	trace
Hansenula anomala AKU 4300	4	trace	11.70	2.60
	8	1.60	14.80	5.36
	12	2.60	10.80	4.86
Hansenula jadinii IFO 0987	4	7.00	4.60	3.12
	8	7.20	6.20	1.68
	12	7.12	5.88	1.68

TABLE III. DISTRIBUTION OF CDP-CHOLINE FORMING ACTIVITY IN YEASTS (continued)

Strains	Reaction time (hr)	Products and substrate(μmoles/ml)		
		CDP-choline	5'-CMP	CDP + CTP
Hansenula capsulata IFO 0721	4	4.40	2.36	12.80
	8	5.00	6.80	12.00
	12	5.20	10.40	8.60
Debaryomyces japonicus IFO 0039	4	0	8.20	3.60
	8	0	6.24	2.08
	12	0	6.60	4.88
Debaryomyces hansenii IFO 0023	4	5.20	1.70	10.10
	8	8.20	1.76	9.28
	12	10.00	1.80	7.60
Debaryomyces globosus IFO 0016	4	5.28	4.80	5.40
	8	10.00	2.48	8.20
	12	11.80	6.56	10.70
Debaryomyces subglobosus IFO 0794	4	6.32	2.48	12.00
	8	10.40	3.00	8.84
	12	11.40	3.48	8.20
Debaryomyces cantarellii IFO 1189	4	trace	15.20	1.08
	8	1.22	19.80	2.72
	12	1.40	12.80	8.32
Debaryomyces castellii IFO 1359	4	trace	15.90	7.32
	8	trace	17.20	9.00
	12	1.80	17.20	8.28
Debaryomyces coudertii IFO 1381	4	1.60	15.80	0.56
	8	2.60	12.40	2.20
	12	2.60	12.10	2.96
Debaryomyces vini AKU 4356	4	8.80	1.88	7.80
	8	12.60	4.08	7.60
	12	13.80	10.40	2.20
Saccharomycodes ludwigii AKU 4400	4	trace	2.60	17.80
	8	trace	4.20	17.60
	12	trace	4.40	17.60
Hanseniaspora valbyensis IFO 0115	4	trace	2.72	20.40
	8	trace	6.40	16.20
	12	trace	3.80	15.60

TABLE III. DISTRIBUTION OF CDP-CHOLINE FORMING ACTIVITY IN YEASTS (continued)

Strains	Reaction time (hr)	Products and substrate (μmoles/ml)		
		CDP-choline	5'-CMP	CDP + CTP
Nadsonia elongata IFO 0665	4	trace	14.20	5.80
	8	trace	11.60	10.80
	12	trace	11.40	8.00
Lipomyces lipoferus IFO 0673	4	3.72	10.30	3.04
	8	5.12	3.08	9.00
	12	6.12	6.48	9.20
Sporobolomyces roseus IFO 1037	4	1.36	9.40	trace
	8	1.72	8.00	trace
	12	2.20	7.40	trace
Sporobolomyces roseus IFO 1105	4	trace	11.50	1.28
	8	trace	12.70	2.00
	12	trace	10.80	0.96
Sporobolomyces salmonicolor IFO 0374	4	2.48	6.40	13.00
	8	4.00	10.60	2.60
	12	7.20	7.20	4.40
Trigonopsis variabilis IFO 0671	4	trace	4.32	12.20
	8	2.40	4.88	13.90
	12	2.40	19.80	2.52
Cryptococcus albidus IFO 0378	4	0.80	9.40	5.36
	8	2.28	6.20	4.80
	12	2.88	4.12	2.00
Torulopsis candida IFO 0768	4	6.20	1.60	12.40
	8	11.80	2.40	8.80
	12	15.10	3.20	7.60
Torulopsis sphaerica IFO 0648	4	1.48	2.00	6.72
	8	2.68	4.00	6.28
	12	3.12	2.00	12.00
Torulopsis famata IFO 1084	4	0	12.80	trace
	8	0	7.20	trace
	12	0	3.20	trace
Rhodotorula glutinis IFO 0389	4	1.00	11.60	2.72
	8	2.00	12.70	4.32
	12	1.60	8.00	3.12

TABLE III. DISTRIBUTION OF CDP-CHOLINE FORMING ACTIVITY IN YEASTS (continued)

Strains	Reaction time (hr)	Products and substrate (μmoles/ml)		
		CDP-choline	5'-CMP	CDP + CTP
Brettanomyces claussenii IFO 0627	4	2.80	3.40	16.40
	8	4.28	6.20	17.00
	12	8.68	4.20	19.40
Candida utilis IFO 0396	4	5.20	16.10	trace
	8	5.40	19.00	trace
	12	4.68	16.80	trace
Candida humicola IFO 0760	4	0	12.30	trace
	8	0	10.40	trace
	12	0	8.08	trace
Candida krusei IFO 0013	4	8.20	2.24	12.00
	8	8.20	13.50	3.28
	12	7.48	13.20	4.20
Candida tropicalis IFO 0006	4	6.16	trace	11.80
	8	8.00	3.40	8.52
	12	7.00	4.08	9.40
Candida intermedia IFO 0761	4	0.80	10.00	1.60
	8	2.28	6.60	1.56
	12	2.88	5.44	2.16
Candida parapsilosis IFO 0708	4	5.86	1.60	9.44
	8	7.20	5.00	7.80
	12	6.40	8.20	6.52
Candida pseudotropicalis IFO 0617	4	7.20	5.08	8.60
	8	9.60	6.88	9.00
	12	8.60	9.20	11.80
Kloeckera apiculata IFO 0154	4	0	6.20	0
	8	0	2.68	0
	12	0	2.20	0
Baker's yeast	4	5.28	3.28	11.00
	8	12.60	3.12	8.60
	12	10.60	6.20	8.60

SUMMARY

Large amounts of a cytidine derivative were formed when air-dried cells of brewer's yeast (100 mg/ml) were incubated with 5'-CMP (20 μmoles/ml) and phosphorylcholine (50 μmoles/ml) in the presence of glucose and phosphate buffer. The cytidine compound was isolated from the reaction mixture by ion exchange column chromatography with Dowex 1 x 2 (Cl^- form) and identified as CDP-choline by several enzymic and physicochemical methods.

The effects of several factors affecting CDP-choline fermentation by brewer's yeast were investigated with respect to reaction pH and concentrations of substrates.

The distribution of CDP-choline accumulating activity was tested among various yeasts other than brewer's yeast. Out of 43 strains, 33 strains showed the ability to form CDP-choline, and 8 strains transformed 5'-CMP to CDP-choline in more than 50% yield. *Saccharomyces rouxii* IAM 4309 and *Zygosaccharomyces soja* (*Saccharomyces rouxii*) IFO 0495 converted about 80% of 5'-CMP to CDP-choline.

Section 2. Fermentative Production of CDP-Ethanolamine

INTRODUCTION

CDP-Ethanolamine is an analogous cytidine coenzyme to CDP-choline and was first found by Kennedy and Weiss[78] to be an important intermediate in the biosynthesis of phosphatidylethanolamine. In the previous section, the author has described the preparative method of CDP-choline from 5'-CMP and phosphorylcholine with air-dried cells of brewer's yeast. This section deals with the production of CDP-ethanolamine by the application of the fermentative method of CDP-choline.

MATERIALS AND METHODS

Materials. Authentic CDP-ethanolamine was purchased from Boehringer and Soehne GmbH, Mannheim. All other chemicals used in this study were the same as described in the previous section.

Microorganism. Brewer's yeast obtained from Kirin Brewery Co., Ltd. was air-dried by the method described in the previous section. The dried cells were used for CDP-ethanolamine formation as enzyme source.

Analyses. Cytidine compounds including 5'-CMP, CDP, CTP and CDP-ethanolamine were determined by paper chromatography as described in the previous section. Anion exchange column chromato-

graphy with Dowex 1 x 2 (formate form) was carried out for the separation of CDP-ethanolamine from the reaction mixture. The stepwise elution was performed with formic acid-ammonium formate system. Before applying to the column, the reaction mixture was concentrated by treatment with charcoal, followed by elution with ethanol-NH_4OH-H_2O (50:5:45, v/v) mixture. Inorganic phosphate was determined by the method of Fiske and Subbarow[73] after degradation of isolated CDP-ethanolamine with sulfuric acid and various enzymes. Phosphorylethanolamine was determined colorimetrically as DNP-derivative by the method of Nojima and Utsugi.[85]

Reaction system. The composition of the reaction mixture for CDP-ethanolamine formation is shown in Table I. The reaction was terminated by immersing the tube in boiling water for 3 min and the supernatant solution was submitted to analyses.

TABLE I. COMPOSITION OF REACTION MIXTURE

	per ml
5'-CMP sodium salt	20 μmoles
Phosphorylethanolamine	50 μmoles
Potassium phosphate buffer (pH 7.4)	200 μmoles
Glucose	400 μmoles
Calcium chloride	50 μmoles
Air-dried cells of brewer's yeast	100 mg

Total reaction mixture of 2.0 ml was put in a test tube and reaction was carried out at 28°C with shaking.

RESULTS AND DISCUSSION

Conversion of 5'-CMP to CDP-ethanolamine by brewer's yeast

After incubation with the reaction mixture shown in Table I, the products were analyzed by paper chromatography. As is shown in Fig. 1, an ultraviolet absorbing spot having the same *Rf* value as authentic CDP-ethanolamine was detected with incubation time.

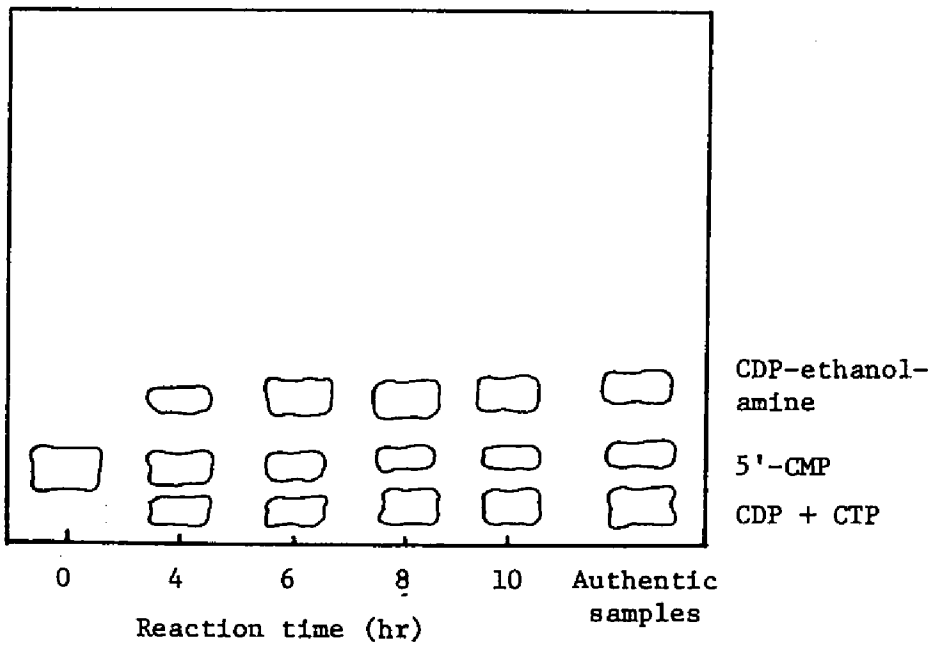

Fig. 1. Formation of CDP-Ethanolamine from 5'-CMP by Dried Cells of Brewer's Yeast.

The reaction system was the same as that described in Table I. At the time indicated above, an aliquot of the reaction solution was submitted to analysis by paper chromatography with 95% ethanol-NH_4OH-M ammonium acetate (10: 4: 1, v/v).

Isolation and identification of CDP-ethanolamine

In order to isolate and identify this compound, a large scale incubation was carried out. The reaction mixture contained 2 mmoles of 5'-CMP sodium salt, 20 mmoles of phosphorylethanolamine, 20 mmoles of potassium phosphate buffer (pH 7.4), 120 mmoles of glucose, 10 mmoles of calcium chloride and 20 g of dried cells brewer's yeast in a final volume of 200 ml. The reaction was carried out at 28°C in a 2 liter-flask with continuous shaking. After incubation for 10 hr, the flask was immersed in boiling water for 5 min and cooled. The cells were centrifuged off, and to the supernatant solution (total optical density at 260 mμ was 3×10^4) was added 15 g of charcoal and stood for 60 min with stirring. Then, the solution was filtered and the charcoal was washed with 0.001 N HCl. The adsorbed nucleotides were eluted with 50% ethanol containing 5% NH_4OH. The eluate was concentrated at 30°C to a small volume under reduced pressure. It was adjusted to pH 8.0 with NaOH and applied to a column of Dowex 1 x 2 (formate form, 2.5 x 100 cm, resin volume 340 ml). The stepwise elution was carried out with formic acid-ammonium formate system. The elution pattern of the nucleotides is shown in Fig. 2. Each fraction was analyzed by paper chromatography and found to be as follows: A, CDP-choline; B, CDP-ethanolamine compound; C, 5'-CMP; D, CDP + CTP. Then the fraction (B) was collected and treated again with charcoal as described above.

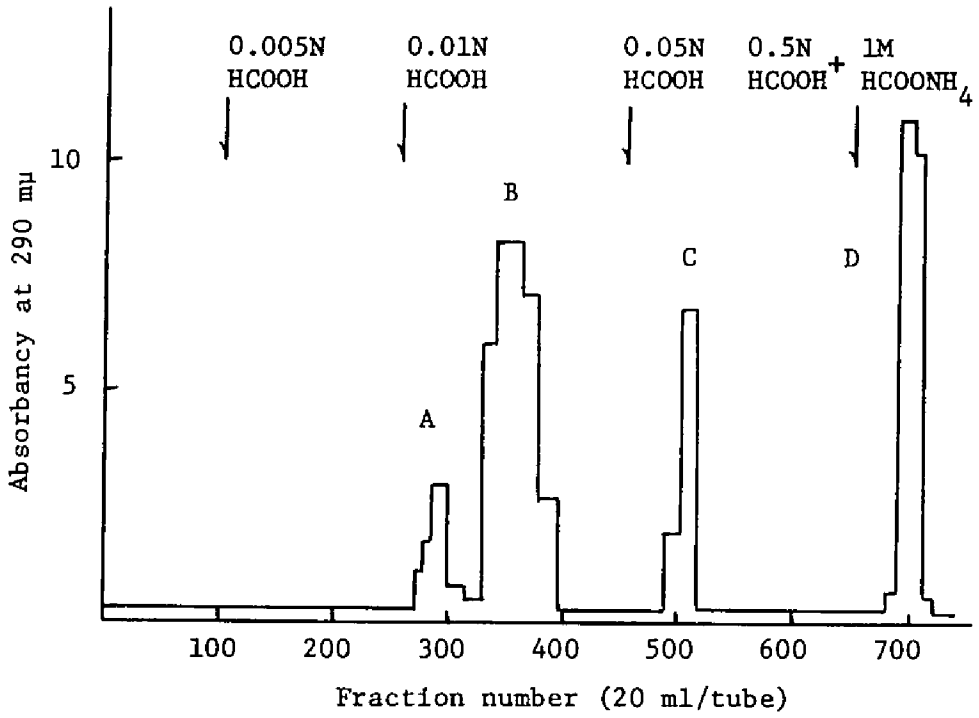

Fig. 2. Separation of CDP-Ethanolamine by Dowex 1 x 2 (Formate Form) Column Chromatography.

Column size : 2.5 x 100 cm, Bed volume: 340 ml

The eluate with ammoniacal ethanol solution was concentrated by evaporation at 30°C under reduced pressure and lyophilized. Finally, about 200 mg of lyophilized powder was obtained. The powder was used for the identification for CDP-ethanolamine.

Paper chromatography of the isolated cytidine derivative and its acid-hydrolyzate was carried out with three kinds of solvent systems. As is shown in Table II, the *Rf* value of the isolated sample was almost identical with that of authentic CDP-ethanolamine both in UV-absorbing and ninhydrin-positive spots. Hydrolysis

TABLE II. PAPER-CHROMATOGRAPHY OF ISOLATED CDP-ETHANOLAMINE AND ITS ACID-HYDROLYZATE

	Rf values		
	Solvent I	Solvent II	Solvent III
UV-absorbing spot			
CDP-ethanolamine	0.38	0.17	0.48
5'-CMP	0.25	0.24	0.57
Sample	0.38	0.17	0.48
Hydrolyzate	0.25	0.24	0.55
Ninhydrin-positive spot			
CDP-ethanolamine	0.38	0.17	0.48
Phosphorylethanolamine	0.37	0.28	0.60
Ethanolamine	0.66	0.54	0.57
Sample	0.38	0.17	0.48
Hydrolyzate	0.37	0.28	0.58

Solvent I: 95% ethanol-M ammonium acetate (7.5: 3, pH 7.5)
Solvent II: 0.02 N acetic acid in 60% ethanol
Solvent III: Isobutyric acid-0.5 N NH_4OH (5:3, v/v)

of the sample in 1 N HCl at 100°C for 40 min gave two compounds; one was identical with 5'-CMP, and the other was identical with authentic phosphorylethanolamine by paper chromatography. It was also found that equimolar amount of 5'-CMP and phosphorylethanolamine was released from one mole of the sample by the hydrolysis. Table III shows the results of phosphate analysis. Two moles of phosphorus were released from one mole of the sample after decom-

position by sulfuric acid. When acid-hydrolyzate (1 N HCl, 100°C, 40 min) of the sample was treated with bull seminal 5'-nucleotidase, one mole of phosphorus and one mole of cytidine were liberated. On treatment of the sample with snake venom (*Agkistrodon halys*, Mamushi),[82] which contained nucleotide pyrophosphatase and 5'-nucleotidase, the sample liberated one mole of phosphorus. These data show that the isolated cytidine derivative is composed of 5'-CMP and phosphorylethanolamine, which are bound through pyrophosphate linkage.

TABLE III. PHOSPHATE ANALYSIS OF ISOLATED CDP-ETHANOLAMINE

	Pi found* (μmoles)	Theoretical value
Total phosphates	2.03	2.00
Treatment with 5'-nucleotidase after acid-hydrolysis**	0.93	1.00
Treatment with snake venom (nucleotide pyrophosphatase and 5'-nucleotidase)	1.08	1.00

* μmoles per μmoles of isolated CDP-ethanolamine

** 1 N HCl, 100°C, 40 min

The IR and NMR spectra of the isolated sample are shown in Fig. 3 and Fig. 4. The IR spectrum of the sample was identical with that of authentic CDP-ethanolamine. The signals in the NMR spectrum showed the presence of protons of H-5 and H-6 in pyrimi-

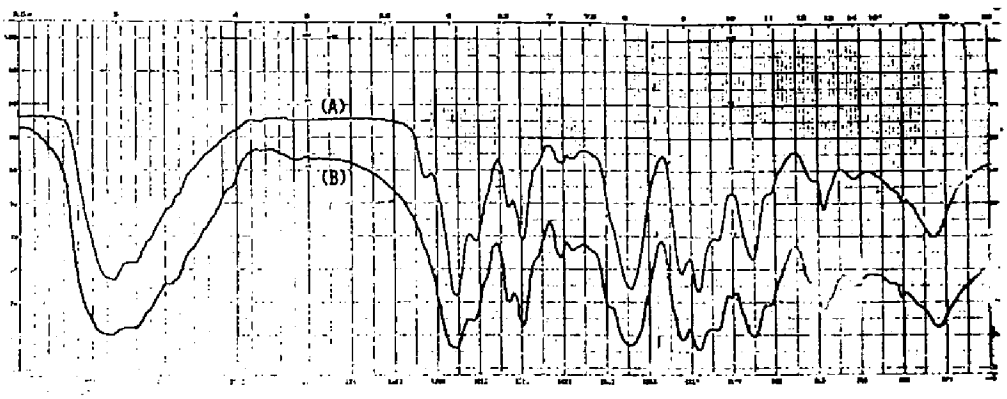

Fig. 3.. IR Spectra of Isolated and Authentic CDP-Ethanolamine.

(A): Isolated CDP-Ethanolamine, (B): Authentic CDP-Ethanolamine

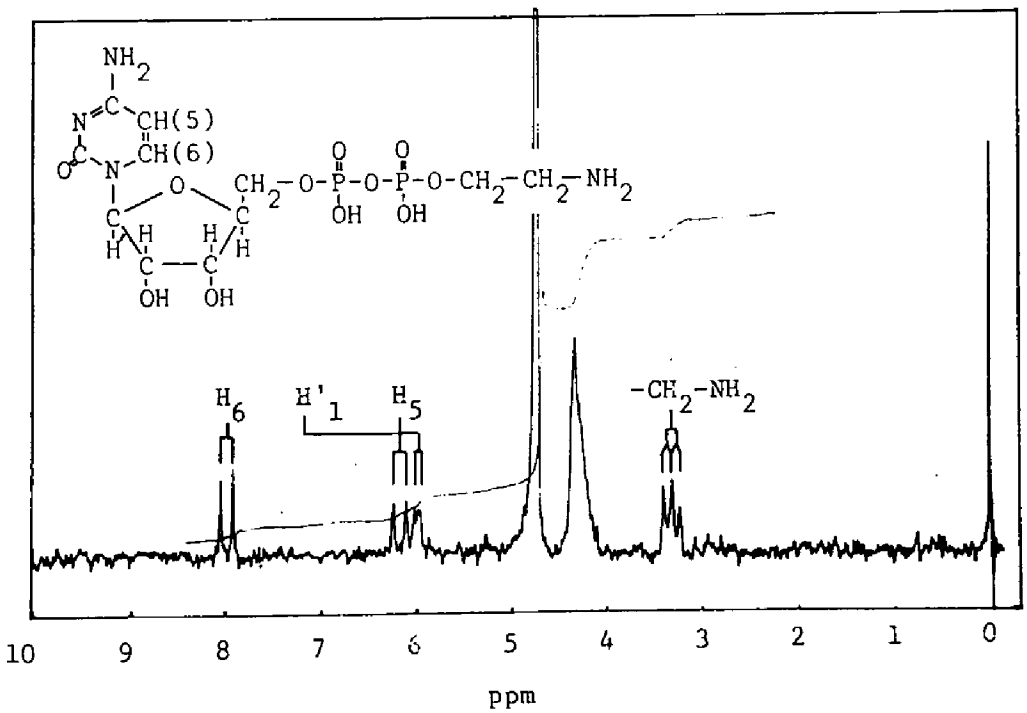

Fig. 4. NMR Spectrum of Isolated CDP-Ethanolamine.

dine base, H-1' in ribose, and two protons attached to the terminal carbon atom of ethanolamine residue.

Anal. Found C, 23.89; H, 4.51; N, 10.10%. Calcd. for $C_{11}H_{18}O_{11}N_4P_2Ca \cdot 4H_2O$: C, 23.80; H, 4.68; N, 10.08%.

From the results described above, it was concluded that the isolated cytidine compound was CDP-ethanolamine. A typical time course of CDP-ethanolamine formation from 5'-CMP and phosphoryl-ethanolamine by dried cells of brewer's yeast is shown in Fig. 5.

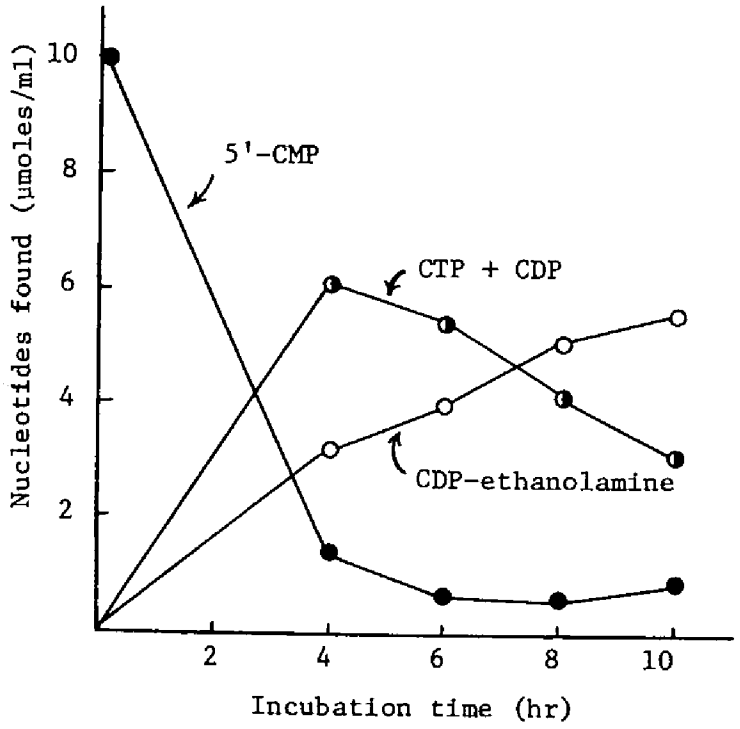

Fig. 5. Time Course of Formation of Cytidine Derivatives. The reaction system was the same as that described in Table I except that 10 μmoles/ml of 5'-CMP was incubated.

Similarly to the fermentative process of CDP-choline described in the previous section, 5'-CMP was rapidly converted to CDP and CTP in the earlier stage of the reaction, followed by gradual accumulation of CDP-ethanolamine. The cytidine coenzyme was accumulated with about 55% yield based on 5'-CMP after incubation for 10 hr.

SUMMARY

CDP-Ethanolamine, an analogous cytidine coenzyme to CDP-choline, was formed when the air-dried cells of brewer's yeast were incubated with 5'-CMP, phosphorylethanolamine, phosphate buffer, glucose and calcium ion. The cytidine coenzyme was isolated from the reaction mixture by ion exchange column chromatography with Dowex 1 x 2 (formate form). The compound was identified as CDP-ethanolamine by paper chromatography and phosphate analyses. It was also demonstrated by several enzymic and physicochemical methods. CDP-Ethanolamine was accumulated with about 55% yield based on 5'-CMP after incubation for 10 hr.

CONCLUSION

In this study, the author has investigated the fermentative production and metabolism of biologically important pyrimidine nucleoside diphosphate coenzymes by yeasts.

In the first chapter, it was described that large amounts of UDPGal were accumulated when the air-dried cells of *Torulopsis candida* IFO 0768 grown on a galactose or lactose medium were incubated aerobically with 5'-UMP and galactose (or lactose) in the presence of high concentration of inorganic phosphate. UDPGal was isolated from the reaction solution by treatment with charcoal and ion exchange column chromatography, and identified by several methods. Various factors on UDPGal fermentation by the yeast were investigated, the maximal yields being 60-80% when the fermentative conditions were optimized. By investigating the distribution of UDPGal forming activity in various yeasts, *T. candida* was found to be the most suitable strain for UDPGal accumulation.

One of the most important factors that affected the fermentative production of UDPGal was the water content of the dried cells used as enzyme source. Little accumulation of UDPGal occurred by the use of the lactose-grown dried cells having more than 20% water. It was essential for a maximal accumulation of UDPGal to use well-desiccated cells having less than 10% water, in contrast to the observations in UDPG formation by glucose-grown cells. It was

concluded that the above effect on UDPGal fermentation might be brought about by the changing degree of excretion of the enzymes responsible to UDPGal synthesis from dried cells of different water contents.

The enzyme activities which were responsible to the synthesis of UDPGal by lactose-grown *T. candida* were investigated with the cell-free extract and ammonium sulfate fraction. The presence of galactokinase, Gal-1-P uridylyltransferase, UDPGal 4-epimerase and UDPG pyrophosphorylase was demonstrated, but UDPGal pyrophosphorylase was very weak. It was elucidated with the cell-free system that the formation of UDPGal proceeded by a coupling reaction with UDPG pyrophosphorylase and Gal-1-P uridylyltransferase, in which UDPG or G-1-P acted as a catalyst.

It was also observed that the conversion of UDPGal to UDPG by air-dried cells of *T. candida* was remarkably inhibited by the coexistence of 5'-UMP and galactose. This fact strongly suggested that UDPGal 4-epimerase activity of the yeast might be inhibited by 5'-UMP and galactose. In fact, a partially purified UDPGal 4-epimerase from *T. candida* was markedly inhibited by 5'-UMP, provided that galactose was also present. Therefore, it was concluded that the mechanism of UDPGal accumulation under the fermentative condition was due to a concerted inhibition of the epimerase by 5'-UMP and galactose served as substrates for the fermentation.

Furtheremore, UDPGal 4-epimerase of the yeast was found to require exogenous NAD for the full activity, unlike that reported in microbial sources such as *Saccharomyces fragilis* and *Escherichia coli*. The *Kms* were 1.4 x 10^{-4} M for NAD, and 1.2 x 10^{-3} M for UDPGal, respectively. About 75% of the enzyme activity was lost in the presence of 5'-UMP (5 x 10^{-3} M) and galactose (1 x 10^{-1} M). It was also observed that the catalytic activity was almost recovered by a short dialysis of the enzyme preparation which was inactivated by 5'-UMP and galactose. A strong inactivation of the enzyme activity was found with the combination of 5'-UMP and glucose.

In the second chapter, the author has investigated the fermentative production of UDPAG from 5'-UMP and glucosamine with air-dried cells of baker's yeast as enzyme source. It was found that UDPAG was accumulated in good yields when air-dried cells of baker's yeast were incubated aerobically with 5'-UMP, glucosamine and fructose in the presence of inorganic phosphate. UDPAG was isolated from the reaction mixture by means of anion exchange column chromatography and identified by several biochemical methods. The reaction conditions for the production of UDPAG were investigated. The final yield of UDPAG was about 40-66% based on 5'-UMP when the fermentative conditions were optimized. The concentrations of glucosamine and phosphate buffer, and pH as well as the water content of air-dried cells of baker's yeast greatly affected UDPAG fermentation.

The distribution of UDPAG forming activity in other yeasts was investigated under the same reaction conditions as employed with baker's yeast. It was found that several *Debaryomyces* species showed relatively strong activity of UDPAG formation with 33-40% yields.

By the application of the fermentative production of uridine coenzymes described in the preceding chapters, the author has investigated in the last chapter the preparative method for CDP-choline and CDP-ethanolamine from 5'-CMP. Large amounts of CDP-choline were formed when air-dried cells of brewer's yeast were incubated with 5'-CMP and phosphorylcholine in the presence of glucose and phosphate buffer. CDP-Choline was isolated from the reaction mixture by anion exchange column chromatography and identified by enzymic and physicochemical methods. The effects of several factors affecting CDP-choline fermentation were studied. About 80% of 5'-CMP was converted to CDP-choline under the optimal fermentative conditions. It was also found that many strains of yeast tested had the ability to form CDP-choline in good yields, especially two strains of *Saccharomyces rouxii* (IAM 4309 and IFO 0495) converted about 80% of 5'-CMP to CDP-choline.

On the other hand, CDP-ethanolamine was formed when phosphorylcholine was replaced by phosphorylethanolamine in the reaction system for CDP-choline formation. The isolation and identification

of CDP-ethanolamine were performed. The cytidine coenzyme was accumulated with about 55% yield based on 5'-CMP under the reaction condition.

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